ANTIMICROBIAL COMPOUNDS FROM TROPICAL RAINFOREST PLANTS

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For My Parents

The People of West New Britain

Tony Valuka who Died of Malaria 2001

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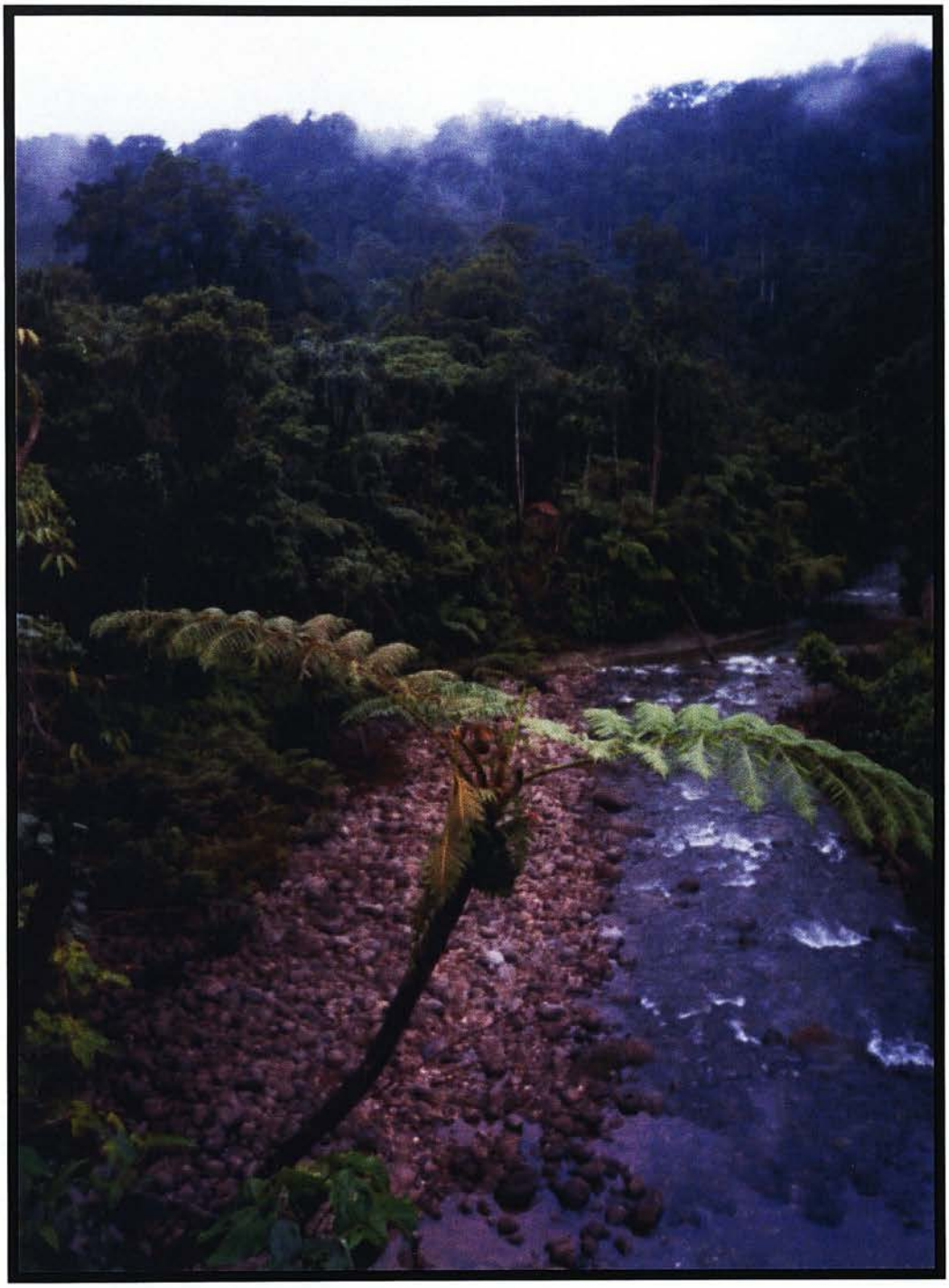
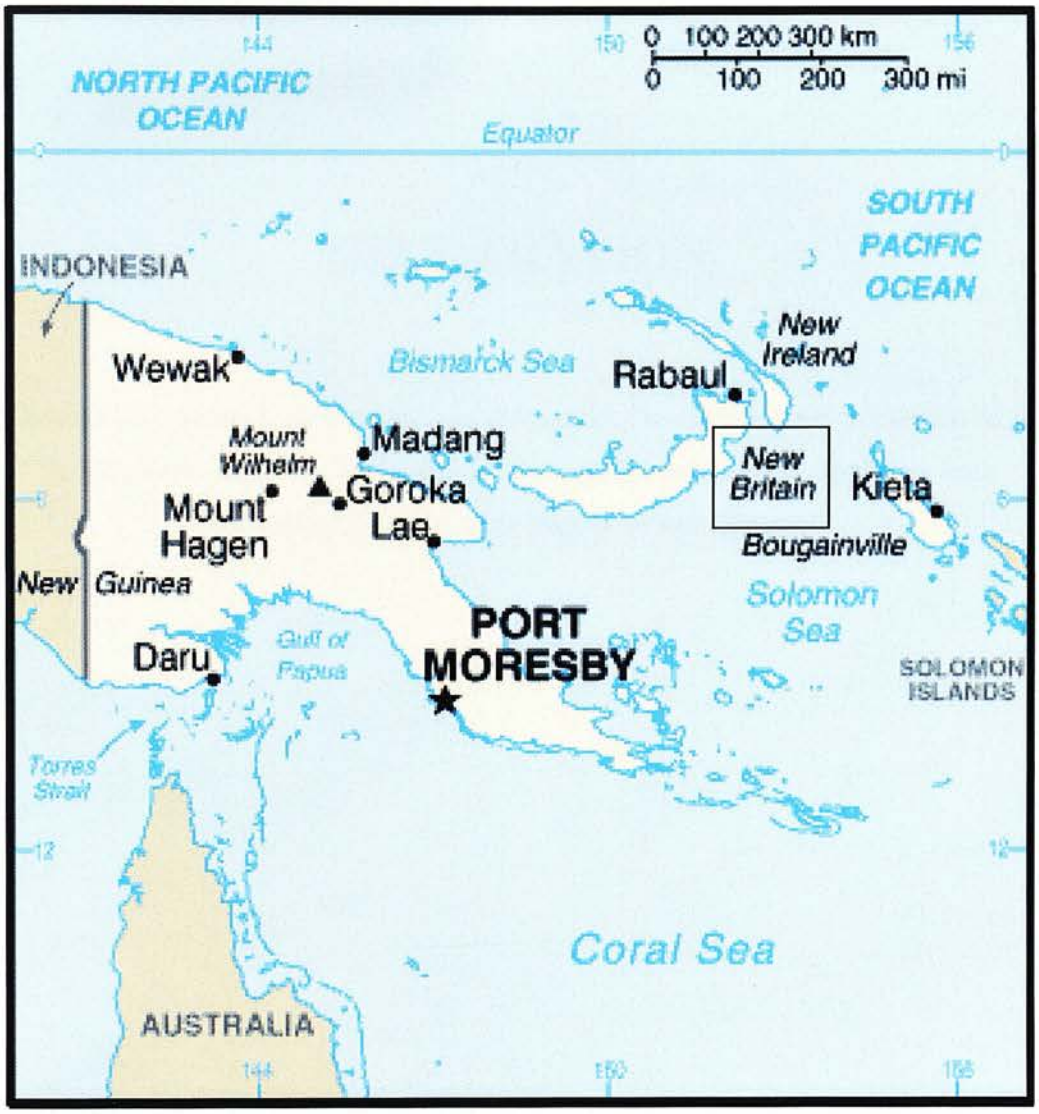
"A country which contains more strange and new and beautiful objects than any other part of the globe" Sir Alfred Russel Wallace on New Guinea, 1858.



Primary rainforest in the Whiteman Range, West New Britain Province, Papua New Guinea



Papua New Guinea, adapted from United States Central Intelligence Agency map

DECLARATION

I Thomas Alexander Kyle Prescott performed all the experiments presented in this thesis unless otherwise indicated in the text. No part of this work has been submitted for any other degree or qualification

AIMS

The aims of the project were as follows.

1. To develop a screening methodology that would allow the efficient identification of tropical rainforest plants with antibacterial activity.

2. To develop methods for purification of the active principles from the most promising plants.

3. Using spectrometric techniques, to chemically characterise the active principles

4. To test the pure compounds on both sensitive and drug-resistant bacterial pathogens along with human cell lines to determine their biological activities.

5. Where possible to determine the mode of action of active principles.

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ABSTRACT

Tropical rainforest plants represent a potential source of novel antimicrobial drugs since they have adapted to their humid, septic environment by synthesising such compounds as a means of self defence. Similarly, people who inhabit rainforests have adopted certain species to treat infectious diseases, and by investigating such plant medicines it may be possible to find novel compounds with therapeutic potential. With this is mind, ethnobotanical fieldwork was carried out in New Guinea, an area of the world little investigated for indigenous plant medicines and rich in endemic flora. An antibacterial field assay kit was developed using freeze-dried strains of *S. epidermidis* and *E. coli* which allowed plants used in traditional medicines to be screened in situ without having to take them back to a laboratory. This approach identified *Lunasia amara* (Blanco) as a candidate species; the use of its bark by tribes of the Whitman Range to treat tropical ulcers, supported by clear zones of inhibition with *S. aureus*. Samples of the bark were collected for analysis and through activity-guided fractionation, the anti- *S. aureus* activity of the bark extract was pinned down to a single well resolved HPLC peak (MIC *S. aureus* NCTC 6571 64μg/ml) which subsequent NMR analysis revealed to be the quinoline alkaloid lunacridine; 2'-*O*-trifluoroacetyl lunacridine was found to be a more stable derivative however. Lunacridine's planar cationic structure suggested it might act as a DNA intercalator; 220μM giving 50% binding in an ethidium bromide displacement assay. This in turn suggested DNA topoisomerase II as a likely target for the compound which was confirmed with a kDNA decatenation assay revealing complete inhibition of the enzyme at 5μM. Cell viability assays with MRC-5, H226 and HELA cells showed the compound to be cytotoxic in a time dependent manner producing non-linear dose response curves indicative of a topoisomerase poison mode of action. Activation of the apoptosis pathway enzymes caspase 3/7 was also detected, reaching maximal activity between 24 and 48 hours for the H226 cell line. Thus, lunacridine does not represent a selective antibiotic but with the right structural modifications could be developed as an antineoplastic agent.

Chapter 1

INTRODUCTION

1.1 Tropical rainforest plants as a source of antimicrobial agents

The defining features of a tropical rainforest climate are high annual rainfall of at least 250cm, temperatures which do not fall below 18°C as the monthly mean and humidity at ground level not far below 100% (Whitmore, 1990). In such conditions bacteria and fungi flourish; so for rainforest plants, adaptation to their environment requires an effective means of self defence from microbial plant pathogens. The synthesis of antimicrobial secondary metabolites represents one method by which plants defend themselves from infection and it is this that has prompted numerous efforts to screen rainforest plants in the hope of discovering novel antibiotics (Florey, 1949; Newton et al., 2000).

A second feature of rainforests that makes them conducive to drug discovery is their biodiversity. It is estimated that there are 250,000-500,000 species of flowering plants on earth and that approximately half of these are found in rainforests (Borris, 1996; Cowan, 1999; Mendelsohn, 1995); one single hectare of tropical rainforest may contain over 100 tree species, more than the total for all of northern Europe (Whitmore, 1990). Thus whilst the British Isles have approximately 1,800 native plant species Peru has closer to 18,000 (Macilwain, 1998). It is reasonable to assume that the floristic biodiversity of tropical rainforests is reflected in the chemical diversity of their constituent plants. Currently the number of structurally elucidated secondary metabolites in the world stands at more than 20,000 and is rapidly increasing (Harborne, 1988) whilst estimates for the total number of secondary metabolites present in plants suggest figures as high as 500,000 (Firn, 1996).

In addition to this only 5-15% of the worlds higher plants have been systematically investigated for pharmacologically active compounds (Balandrin, 1993). Altogether there are 47 clinically used drugs on the market that owe their origins to tropical rainforests. Based on the number of pharmacologically un-evaluated plants and the number of potential screening methods available it has been estimated that at least 300

potential clinically viable drugs remain to be discovered in tropical forests around the world (Mendelsohn, 1995).

Rather than representing a set of random structures the great chemical diversity present in rainforest plants has in fact been honed through many millennia of evolution so that each secondary metabolite may provide a range of functions to a plant such as defence against herbivores, microbes, viruses and competing plants or signals to attract pollinating or seed dispersing animals (Dixon, 2001; Wink, 2003). Thus secondary metabolite structures are crafted through evolution to interact with specific receptors in the target organism (Onaga, 2001). For example certain species of Solanaceae synthesize alkaloids with the same quaternary nitrogen configuration that is present in most neurotransmitters and only this precise configuration allows the alkaloids to interact with its receptor (Wink, 2003). This process has been termed "evolutionary molecular modelling" in analogy to the molecular modelling applied in structure-based drug design (Wink, 2003). The highly specific interactions of some plant-derived antimicrobial compounds suggest they too have been tailored to dock with specific target receptors through years of evolution. Such specific interactions can easily be interrupted however, (Duffy et al., 2003) and since the lifecycle of a microbe is vastly shorter than the plant it infects there is ample opportunity for the microbe to evolve resistance to the compound and a lot less time for the plant to respond. Evidence suggests that plants overcome this problem by synthesising more than one active compound and by keeping a wide range of molecules as stock to synthesise new antimicrobials should the need arise (Firn, 1996). The synthesis of secondary metabolites requires a significant expenditure of raw materials, energy as well as genetic space to code for their biosynthesis, but this effort is more than paid off by the survival advantage such compounds offer to plants under attack (Attardo and Sartori, 2003).

1.2 Ethnopharmacology

The title of this study namely "the isolation of antimicrobial compounds from tropical rainforest plants" gives rise to the obvious question of which tropical rainforest plants to screen for antimicrobial activity. If purely random sampling techniques are used then one could spend an inordinate amount of time assaying plants with no guarantee of finding an active species to work on (Shultes, 1994). Indeed, results of screening natural product-based libraries for antibiotics have yielded hit rates as low as one in 30,000 to 40,000 (Onaga, 2001). What is needed is a way of narrowing down the extensive plant biodiversity of the tropical rainforests to a range of identified plant species associated with a particular antimicrobial activity. One way of doing this is to use local knowledge of rainforest tribes who use plants to treat infections that are common ailments of rainforest life (Xue and Zhang, 1998). For example, a study by (Brandao et al., 1992) of plants used to treat malaria in the Brazilian Amazon found 18% of the 22 plants recorded showed activity whereas a mere 0.7% were active in a study carried out by Carvalho et al in which 273 plants were randomly selected for screening (Elisabetsky and Shanley, 1994). In a second study, plants selected for antiviral screening on the basis of their folklore use were five times more likely to be active than those selected randomly (Vlietinck and Vanden Berghe, 1991). Not only is there an increased chance of finding active compounds with the ethnopharmacological approach but it is said that compounds isolated using this approach are less likely to be toxic since they have already been exposed to humans and have therefore in effect already undergone a very basic form of clinical trial. Caution must be applied to this rationale however since certain toxic effects of plant may only become apparent years after the dose has been taken (de Smet and Rivier, 1989).

In the search for novel antimicrobial compounds the use of such indigenous knowledge may be particularly poignant since the humid rainforest environment not only poses a problem for its constituent plants but for its human inhabitants too. For rainforest dwelling people the slightest cut or graze is a potential hazard since it may rapidly

become infected and if left untreated may develop into a more serious infection (Adriaans and Drasar, 1987). Therefore, just as rainforest plants have adapted to their habitat by evolving biosynthetic pathways that produce secondary metabolites to defend themselves, people have adapted to life in rainforests by discovering which plants possess antimicrobial properties and using them to treat infections.

The study of such native medicines is termed ethnopharmacology, the term was first used as the title of a book by Efron et al (Efron, 1970) and was later defined by Bruhn and Holmstedt (1981) as the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man (Bruhn, 1981). The primary goal of an ethnopharmacological investigation is to verify the pharmacological activity of an ethnomedical treatment (Waller, 1993) and broadly speaking such an investigation should proceed in a three step process (de Smet and Rivier, 1989):

1. Botanical or zoological identification of the source material
2. Chemical isolation and identification of the active principles
3. Pharmacological testing of the active principles

Ethnopharmacology is a highly interdisciplinary subject encompassing diverse areas of study such as ethnobotany, chromatography, analytical chemistry and various biomedical disciplines such as pharmacology, microbiology, immunology etc (Waller, 1993; Wickberg, 1993). By using such a broad interdisciplinary approach it is possible to identify plants that are used traditionally to treat specific diseases and then determine if they contain bio-active compounds to provide an explanation of the plants use. It is not possible for a single researcher to master all of these specialities but it is none the less important for each member of an ethnopharmacological research team to have an understanding of each others disciplines.

The first step in any ethnopharmacological investigation is the gathering of field data (Wickberg, 1993) and where the source material is of plant origin the science of

ethnobotany possesses the relevant techniques to allow the acquisition of reliable information.

1.2.1 Ethnobotany

The term ethnobotany was first coined by the Botanist Dr. John Harshberger at a conference in 1895 which he defined as "the study of plants used by primitive and aboriginal people" (Harshberger, 1895; Heinrich, 2001). Thus ethnobotany could be said to be a fusion of botany and anthropology where anthropological techniques are used to gather information on the uses of plants and taxonomic botany is then used to identify the plants (Wickberg, 1993).

Ethnobotany provides the source material along with descriptive information of the medicinal uses of the plant from which all further work in an ethnopharmacological investigation depends, so it is vital that accurate information is obtained (de Smet and Rivier, 1989). The taxonomic identification of plants is important since active compounds may have already been identified from a related species or from members of the same genus since particular types of secondary metabolites tend to predominate within a single taxon (Waller, 1993; Wink, 2003). Misidentification of a plant or insufficiently detailed field observations could severely hamper the chances of verifying the use of the plant later in the investigation.

Understanding the cultural context in which plants are employed is often central to gaining meaningful information on their medicinal use and this is why anthropological skills are required (de Smet and Rivier, 1989). For example, a botanist enquiring about the source material used to prepare a concoction used in rituals may be deliberately supplied with the wrong plants if members of that tribe do not wish to disclose the source of their sacred plant (de Smet and Rivier, 1989). Highly detailed descriptions of the way plants are employed as medicines are vital since the smallest detail may provide

clues as to the disease the plants are intended to treat. For example, plants used to treat weight loss may in fact be intended to treat diabetes since obesity is a symptom of certain types of diabetes (de Smet and Rivier, 1989).

Thus developing a good rapport with members of local communities is vital to gaining reliable ethnobotanical information (Etkin, 1993) and this is most effectively achieved by participating in activities such as collecting plants observing rituals and general situations in which plants are discussed, prepared or used (Etkin, 1993). Such participant observation helps establish rapport with native communities and enables a more precise understanding of the ways in which plants are used. Ethnobotanical information may come from key figures within a native community who are especially involved with administering plant medicines or perhaps more reliably from a number of sources within a community taking only that information which is corroborated by a second source. Despite the purely qualitative nature of such work, ethnobotany is nonetheless the starting point for most ethnopharmacological investigations and is capable of providing meaningful information relating to a specific pharmacological activity. For example, when inhabitants of the Sibundoy Valley in Columbia were asked to rank cultivars of *Brugmansia* according to their toxicity the five cultivars named were found to contain increasing concentrations of the toxic alkaloid scopolamine (de Smet and Rivier, 1989).

1.2.2 Chemical isolation of compounds

The isolation of pure compounds from plant material is one of the most technologically challenges steps in the ethnopharmacological process and is a pre-requisite for structural determination by methods such as nuclear magnetic resonance spectroscopy (NMR) (Wickberg, 1993). Since plants synthesise compounds in quite convoluted pathways a single extract may contain several active principles of similar structure along with inactive but highly related compounds. This means that as the purification process

proceeds it becomes increasingly difficult to separate the active constituents since each chromatography step produces a mixture of increasingly related compounds.

Before separation of the plant analytes can begin it is necessary to determine the optimum extraction solvent in which to solublize the active principles. For plants with antimicrobial activity, ethanol and methanol are popular solvents since the majority of plant antimicrobial compounds are organic heterocycles and are readily soluble in these alcohols (Cowan, 1999). An optimum solvent is one that completely extracts all the active principles but leaves as much of the inactive material behind. Where active principles exhibit a broad range of hydrophobicities more than one extraction solvent may be needed. At the same time a convenient biological assay for the activity in question is needed to determine which solvents extract the most activity and to test active fractions during the purification process.

In order to isolate pure compounds, chromatography techniques are required that provide excellent separating power, are non-destructive, preparative rather than purely analytical and allow at least mg quantities of sample to be run at a time since a minimum of 5mg are needed for NMR and active principles may form only a small proportion by weight of the total material in a plant extract. A variety of techniques fit this description such as thin layer chromatography (TLC), supercritical fluid chromatography (SCFC), counter current chromatography (CCC) and high performance liquid chromatography (HPLC) (Phillipson, 2003).

Each technique offers different advantages, TLC is relatively cheap compared to the other techniques but does not give as good separation. CCC is able to handle gram quantities of sample on relatively cheap apparatus but does not offer as good a separation of some analytes as HPLC (Wickberg, 1993). Overall the choice of technique depends very much on the type of active compounds in the plant and for this reason an accurate identification of the voucher specimen collected in the fieldwork period is vital since a literature search may inform the chromatographer of the classes of compounds present in the sample and direct his choice of technique accordingly (Waller, 1993).

In addition to the techniques described above there are a variety of older techniques that use the traditional open column format that are of use as preliminary steps in a purification process and in some circumstances may be able to replace the more sophisticated high cost techniques. A variety of separation media are available such as silica and charcoal for adsorption chromatography, various cyclodextrans for gel filtration and cellulose based media for ion-exchange. Again the choice of media depends entirely on the analytes in the sample mixture. Care must be taken however since even with standard media such as silica and aluminia it is rare to have 100% recovery of active principles (Wickberg, 1993). Despite this, such techniques are cheaper than their modern counterparts such as HPLC and offer the possibility of scaling up to the gram level without a significant increase in cost.

1.2.3 Identification of compounds

The structural characterisation of active compounds has advanced dramatically in recent years due to the advent of technologies such as NMR and mass spectroscopy which allow structures to be unequivocally solved in a matter of days where they would have once taken years (Wolfender et al., 2003). The development of soft ionisation techniques and instrumental configurations in which HPLC and gas chromatography (GC) are connected directly to mass spectroscopy has increased the ease with which individual molecules can be purified and provided a means by which previously isolated compounds may be identified provided authentic samples are available for comparison (Wolfender et al., 2003). One of the advantages of tandem techniques such as GC-MS and LC-MS is the small quantities required to identify the presence of particular metabolite. The ability to detect sub-microgram quantities is well suited to ethnopharmacological research where an active substance may form only a tiny fraction of the total material in a crude extract.

NMR is a technique that has revolutionised natural product research perhaps more than any other and has therefore impacted greatly on ethnopharmacology too (Phillipson, 2003). With as little as 5mg of a 90% pure compound it is possible to run a proton spectra in a matter of minutes and when combined with C^{13}-spectra and molecular formulae obtained with mass spectrometry a complete characterisation of an active principle is possible. NMR has improved dramatically since its conception; where once 100MHz machines were state of the art 600MHz apparatus with a concomitantly improved resolution are increasingly commonplace and more recently LC-NMR has become available making de-replication of complex samples (distinguishing between already known and novel compounds) a far more efficient process (Wolfender et al., 2003)

1.2.4 Biological activities and testing

The purification and testing of compounds is dependent on a functional assay that is both simple and convenient enough to allow large numbers of semi-pure fractions to be tested (Wickberg, 1993). The choice of assay must be informed by the traditional use of the plant as recorded in the original field observations and must be sufficiently broad so as to encompass the greatest possible number of modes of action within the suspected disease state. A great strength of ethnopharmacology is its potential to deliver compounds with entirely novel modes of action and so inform the wider biomedical community of new drug targets. Thus the microbiologist in an ethnopharmacological investigation must be open to the possibility that the active principles are acting by an entirely new mode of action (Waller, 1993). If the choice of assay is too focussed, compounds with novel modes of action will display no activity at all but if too all encompassing such as in vivo animal models, will not be sufficiently convenient for activity-guided purification of active principles. In practice several assays must be used starting with a simple reproducible test in the purification and finishing with more sophisticated assays once pure compounds have been isolated.

One of the advantages of microbiology based ethnopharmacology i.e. investigations into antibacterial type uses of plants is that rapid and convenient methods for detecting antibacterial activity such as the Kirby Bauer disc diffusion assay already exist and are ideally suited to assaying large numbers of semi-pure fractions. Once pure compounds are available more detailed assays such as minimal inhibitory concentrations (MIC) and time kill assays may be used. Where the ethnobotanical use of a plant suggests an antiviral activity the available methods are not so straight forward since they require the culture of mammalian cell lines.

Viral inhibition may be detected by reduced plaque formation, transformation of cell lines with a colour linked gene or detection of viral products such as surface glycoproteins or reverse transcriptase activity (Cowan, 1999). Because of the requirement to use mammalian cells to culture viruses there is less scope for testing crude plant extracts since compounds in the mixture may have effects on the cells and produce false results. With bacteria and fungi the testing of crude plant extracts is common place and in some cases may be preferable since some plant metabolites work synergistically and show little activity when assayed individually (Cowan, 1999). The types of assays used to test anti-viral activity are sufficiently broad that they routinely detect compounds that act as inhibitors of viral attachment and not just compounds that attack the virus directly; this is a limitation of many studies with antibacterial/fungal plant extracts in that most look only at the growth inhibitory activity (Cowan, 1999).

1.3 A history of plant derived antimicrobial compounds

By identifying plants used by native rainforest populations a variety of useful compounds have been identified some of which have become adopted as clinical treatments for certain infectious diseases. The best known examples are the antimalarial drugs quinine and artemisinin along with the anti-amoebal compound emetine. But whilst there are many noteworthy examples of plant derived anti-protozoal compounds and a plethora of anti-tumour compounds of plant origin there are far fewer examples of antibacterial or antiviral plant derived compounds that have been developed as successful pharmaceutical agents (Cowan, 1999). This section briefly details the history of the development of the better known antimicrobial drugs derived from rainforest plants along with some of the more promising plant-derived drug leads currently undergoing development.

1.3.1 Plant derived anti-protozoal compounds

Plants have proved an excellent source of anti-protozoal drugs; quinine, emetine and the artemisinins all owe their origins to plants and this has resulted in enormous health benefits throughout the world in the treatment of malaria and amoebic dysentery. Among the most remarkable of the three compounds is quinine whose isolation and discovery could be considered one of the first examples of ethnopharmacological drug discovery.

1.3.1.1 Cinchona and the isolation of quinine

Quinine is a quinoline alkaloid found in the bark of *Cinchona* spp. (Rubiaceae) and has potent activity against blood stage malarial parasites (Jaramillo-Arango, 1950). Crucially, it is sufficiently non-toxic to be taken orally and can therefore be considered one of the first systemic antimicrobial compounds. Cinchona species originate in the cloud forests of the Andes from where the bark was brought to Europe once its

antimalarial properties were discovered (Jaramillo-Arango, 1950). The first reference to the cinchona tree comes from Antonio de Calancha who writing in Lima, Peru in 1633 states "There is a tree of fevers in the land of Loja, with cinnamon coloured bark of which the Lojans cast powders which are drunk in the weight of two small coins, and thereby cure fevers and tertians; these powders have had miraculous effects in Lima"(Honigsbaum, 2001).

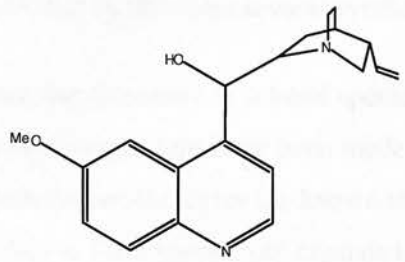


Figure 1.1 Quinine from *Cinchona* spp.

It is not certain that malaria existed in pre-Columbian South America so there is some debate as to whether the discovery of Cinchona's anti-malarial properties should be attributed to the Native American inhabitants or the Jesuits who themselves had a significant interest in medicinal plants. Perhaps the most reliable account of the early use of cinchona bark comes from de Jussieu who writing in 1771 states "it is certain that the discovery is owed to the Malacatos Indians", a tribe from the malarious coastal region of Ecuador who had settled in the valley of Vilcabamba (Honigsbaum, 2001). Since quinine has mild antipyretic properties(Rang, 2003) it seems probable *Cinchona* bark might have been used by local Indians as a febrifuge and then later adapted as a treatment for malaria.

In 1820 the French chemists Pelletier and Caventou became the first scientists to isolate the active principle quinine from Cinchona bark (Jaramillo-Arango, 1950). From the yellow and red barks they obtained a yellow, transparent non-crystalline substance,

which proved to be therapeutically the most active substance present. This substance proved to be a mixture of two alkaloids Cinchonine and Quinine. By removing the acidic fraction from the bark they were able to fractionate the basic portion through partition between immiscible solvents leading to the isolation of pure quinine (Jaramillo-Arango, 1950). After the isolation of quinine there were numerous attempts at a chemical synthesis. The German chemist Runge succeeded in isolating quinoline from coal tar but it was not until 1944 that Woodward and Doering achieved a total synthesis (Woodward, 1944) but by this time several synthetic antimalarials had already been developed.

Since the discovery of several species of plasmodia as the causative agents of malaria, various suggestions have been made for quinines mode of action. Blood stage parasites inhabiting erythrocytes are known to digest haemoglobin and sequester the toxic haem moiety to form haemozoin crystals to prevent the accumulation of free iron which is toxic to the parasite. The most recent studies suggest a mode of action in which antimalarial quinolines bind to the growing crystals either by a surface binding or substrate sequestration mechanism thereby acting as crystallisation inhibitors (Chong and Sullivan, 2003). The resulting build up of toxic haem proves fatal to the parasite. This is backed up by intracellular evidence that shows that quinolines concentrate to millimolar levels in the digestive vacuole, despite circulating in the plasma at nanomolar concentrations (Yayon et al., 1985).

1.3.1.2 Artemisinin from *Artemisia annua*

Artemisinin is an antimalarial compound from the plant *Artemisia annua* (Asteraceae), an annual herb native to Asia, most probably China, where it is known as Qinghao (Balint, 2001). *A. annua* has a long history of use as a medicinal plant in the Far East, the anti-fever properties of Qinghao were first described in 340 A. D by Ge Hong of the East Yin Dynasty in the Zhou Hou Bei Ji Fang (Handbook of Prescriptions for Emergency Treatments). In 1979 reports appeared in the Chinese Medical Journal of a compound isolated from the plant with exquisite activity against blood stage malarial parasites. The active principal artemisinin was revealed to be effective against

chloroquine-resistant strains of *Plasmodium falciparum* which are a factor in the high morbidity rate of malaria world-wide (Balint, 2001).

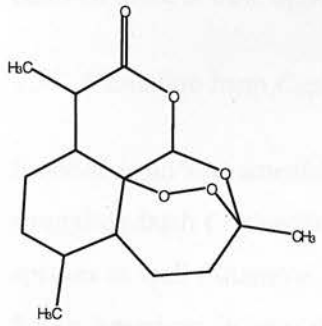


Figure 1.2 Artemisinin from *Artemisia annua*

Artemisinin was isolated using ethyl acetate extraction of the aerial parts of the plant followed by a combination of classical silica column chromatography and crystallisation. Spectroscopic analysis revealed the compound to be a 1, 2, 4-trioxane sesquiterpene lactone (Klayman, 1985) containing an endoperoxide bridge.

The artemisinins are the only class of anti-malarials that remain effective against the drug resistant malarial strains that have emerged since the introduction of chloroquine and artemisinin-based combinations therefore offer a potentially effective way to counter malarial drug resistance. Clinical trials conducted in African children have attested to the good tolerability of oral artesunate when combined with standard antimalarial drugs (Clifford et al., 2003). The clinical efficacy of the drug is characterized by an almost immediate rapid reduction of parasitaemia, and it is an effective stand alone treatment in areas where multidrug-resistance is rampant (Jung et al., 2004).

The mode of action of artemisinin and its derivatives is believed to involve an initial interaction of the endoperoxide moiety with plasmodium sequestered haemozoin, resulting in the formation of free radical intermediates (Avery et al., 2003) which in turn

are highly destructive to a variety of protozoal proteins (Singh and Lai, 2001). By targeting haemozoin the drug offers good selectivity for the parasite since there is no comparable target in human cells, and because its cytotoxic effect occur so swiftly it is claimed there is little opportunity for the parasite to develop resistance.

1.3.1.3 Emetine from *Cephaelis ipecacuanha*

Emetine is an anti-amoebal compound derived from the roots of the South American straggling bush *Cephaelis ipecacuanha* (Rubiaceae) but has been isolated from other species as well (Shamma, 1972). It was used as a treatment for amoebic dysentery by South American Indians (Shamma, 1972) and became widely used as a treatment for dysentery in 18th century Europe. Its close relative dehydroemetine is still used today for the treatment of complicated intestinal amoebiasis and liver abscess caused by *Entamoeba histolytica* (Verhoef, 1986). The name of the plant is derived from an Amerindian word *i-pe-kaa-guéne*, which is said to mean 'road-side sick-making plant' (emetine has emetic properties).

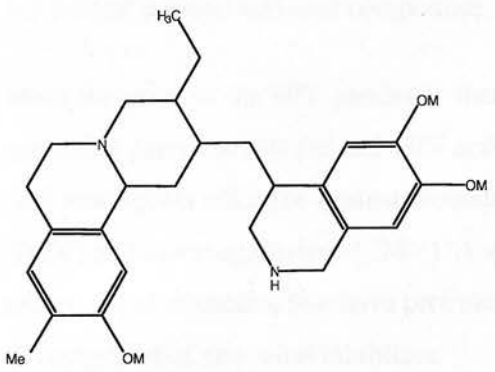


Figure 1.3 Emetine from *Cephaelis ipecacuanha*

M.M Pelletier whose previous work included the isolation of strychnine and quinine applied similar techniques of acidic and basic extraction to partially isolate emetine and

demonstrate it to be responsible for the medicinal properties of Ipecac (Magendie, 1828). Emetine was precipitated from an aqueous extract by the addition of magnesium oxide and then extracted into alcohol before taking up in dilute acid to which purified animal charcoal was added. Trace impurities were then removed by the addition of a salifiable base (Magendie, 1828). The presence of contaminants however meant that the correct empirical formula was not established until 1914 (Shamma, 1972) and the correct structure was not determined until 1948 (Robinson, 1948).

Emetine has been shown to bind reversibly to the amoebic ribosome and therefore act as a protein synthesis inhibitor (Entner and Grollman, 1973). In cell free lysates protein synthesis is inhibited in a dose dependent manner whilst experiments with [^{3}H] emetine revealed an irreversible interaction with free ribosomes which is thought to involve hydrogen bonding via its two nitrogen atoms and a third contact made between the C-1´ carbon atom and the ribosomal chain elongation active site (Entner, 1979).

1.3.2 Plant derived antiviral compounds

Since the onset of the HIV pandemic there have been several programs dedicated to screening plant extracts for anti-HIV activity. There has also been a concomitant need to find new agents effective against secondary viral infections such as herpes simplex virus (HSV) and cytomegalovirus (CMV). A wide array of active compounds have been reported and of these a few have provoked interest as leads compounds for the development of new viral inhibitors.

1.3.2.1 Provir and Virend/SP-303

Virend and Provir are the product names given by Shaman Pharmaceuticals to SP-303, an extract of the sap of *Croton lechleri* (Euphorbiaceae). The tree is a native of the

eastern slopes of the Ecuadorian and Peruvian Andes where it is known locally as Sangre de Grado (Jones, 2003). The sap is used to treat a variety of complaints including HSV infections (Jones, 2003). Provir is indicated for AIDS related diarrhoea whilst Virend which was withdrawn from clinical trials in 1998 due to financial problems was intended for topical treatment of HSV infections. SP-303 is rich in proanthocyanidins such as (-)-galloepicatechin and (+)-gallocatechin with lesser amounts of (+)-epicatechin and catechin (Jones, 2003; Ubillas, 1994). The average molecular weight of the proanthocyanidins in the mixture is approximately 2100Da, each being composed of between 4 and 11 flavanoid units (Jones, 2003).

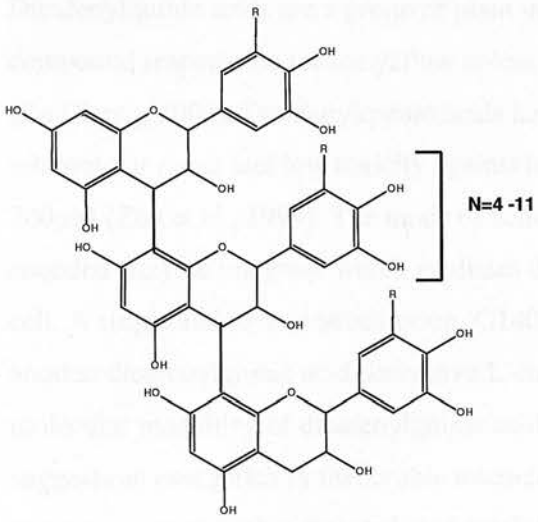


Figure 1.4. Proanthocyanidins (SP-303) from *Croton lechleri*

SP-303 displays activity against a variety of HSV isolates including both HSV-1 and HSV-2 acyclovir resistant strains. It is also inhibitory to respiratory syncytial virus (RSV). In plaque reduction assays, inhibitory concentrations ranged from 0.9 and 2.1μg/ml for various isolates of HSV-2 and 2-10 μM for RSV (Barnard et al., 1993; Jones, 2003). SP-303 was entered into a double-blind, placebo-controlled Phase II

clinical trial for safety and effectiveness against recurrent genital herpes lesions in patients with AIDS. The primary endpoints of the study were complete healing of lesions and time to healing. 41% of patients treated with a topical solution of P-303 experienced complete healing of their lesions compared with 14% in the placebo group (Orozco-Topete et al., 1997). The mode of action of the proanthocyanidins for both viruses is thought to involve inhibition of adsorption early on in the viral life cycle (Barnard et al., 1993; Jones, 2003).

1.3.2.2 Dicafeoylquinic acids

Dicafeoylquinic acids are a group of plant metabolites that include curcumin the compound responsible for the yellow colour of tumeric, *Curcuma longa* (Zingiberaceae) (De Clercq, 2000). Dicafeoylquinic acids have in vitro activity against HIV-1 in the sub micromolar range and low toxicity against human cell lines with LD_{50} values above 700μM (Zhu et al., 1999). The mode of action appears to be inhibition of the virally encoded enzyme integrase which mediates the integration of viral cDNA into the host cell. A single amino acid substitution (G140S) decreased the susceptibility of virus to another dicafeoylquinic acid derivative L-chicoric acid (King and Robinson, 1998) and molecular modelling of dicafeoylquinic acids with the core catalytic domain of integrase suggests an energetically favourable interaction in which the most potent inhibitors occupy a groove within the predicted catalytic site of the enzyme (Robinson et al., 1996). Integrase represents a novel HIV target and as such inhibitors based on the dicafeoylquinic acid structure could be of benefit if used in combination with existing anti-retroviral drugs.

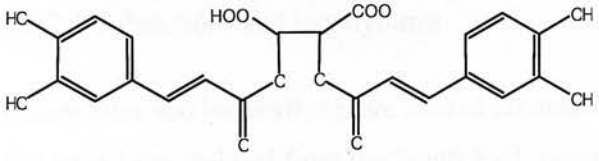


Figure 1.5 L-Chicoric acid from *Echinacea purpurea*

1.3.2.3 Betulinic acid

Betulinic acid is a triterpenoid isolated from *Syzigium claviflorum* (Myrtaceae) and displays HIV-1 inhibitory activity in H9 lymphotic cells with an IC_{50} of 1.4μM. Synthetic structural improvements have produced derivatives of the compound with exquisite activity (inhibition occurring in the sub-nanomolar range) and selectivity index values in excess of 20,000 (Hashimoto et al., 1997). Resistance to betulinic acid derivatives has been mapped to amino acid substitutions at positions 22 and 84 of the HIV antigen gp41. If gp41 is the target then betulinic acid derivatives are the only drug-like small molecules to act as virus-cell fusion inhibitors via gp41and are therefore an ideal template from which to develop new fusion inhibitors (De Clercq, 2000).

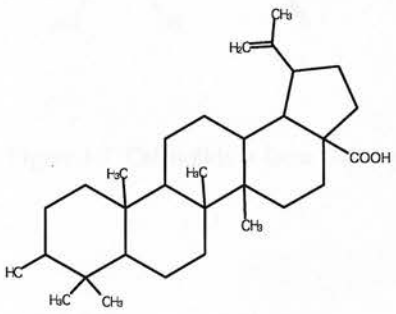


Figure 1.6 Betulinic acid from *Syzigium claviflorum*.

1.3.2.4 Calanolides and inophyllums

Calanolides and inophyllums are related coumarin HIV-1 reverse transcriptase inhibitors that have been isolated from the South East Asian rainforest trees *Calophyllum inopyllum* and *Calophyllum lanigerum* (Clusiaceae) (Kashman et al., 1992) (Patil et al., 1993). The two coumarins have IC_{50} values for reverse transcriptase inhibition of 0.1μ

and 1μM and unlike existing RT inhibitors calanolide analogues have been found to increase their antiviral activity in mutants with the common reverse transcriptase amino acid substitution (Y181C). It therefore seems likely that calanolides and inophyllums bind to HIV-1 reverse transcriptase in a different manner to existing non-nucleoside reverse transcriptase inhibitors which makes them an attractive structural motif from which to develop new leads. The calanolide isomer (+)-calanolide A, is currently being evaluated in clinical trials in the USA (Buckheit et al., 2000).

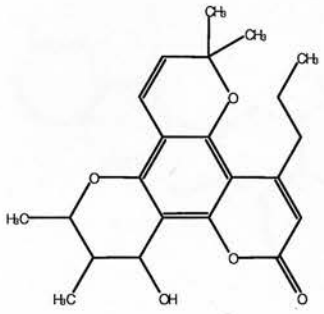


Figure 1.7 Calanolide A from *Calophyllum lanigerum*

1.3.2.5 The camptothecins

Camptothecin, a pentacyclic alkaloid is a topoisomerase 1 inhibitor isolated from the Chinese tree *Camptotheca acuminata* (Nyssaceae). Camptothecin and its analogues have been extensively investigated as potential anti-tumour agents and in 1996 the water soluble variant topotecan was licensed for the treatment of refractory ovarian cancer (Sadaie et al., 2004). Since a number of viruses including HIV and human cytomegalovirus (HCMV) depend upon topoisomerase 1 for DNA replication there has been a certain amount of interest in developing the compounds as viral inhibitors (Bailly, 2000) although concerns over toxicity have so far prevented these compounds from reaching the clinic. One possible advantage of camptothecin based topoisomerase 1

inhibitors is that since the drug target is not virally encoded resistance is expected to develop more slowly than with conventional viral inhibitors. In a single treatment *in vitro* assay a 30nM dose of 9-nitrocamptothecin gave 95% inhibition of HIV-1 replication with minimal cell toxicity suggesting inhibition of topoisomerase I has a greater effect on viral replication than cellular mitosis so providing some degree of selectivity (Balint, 2001).

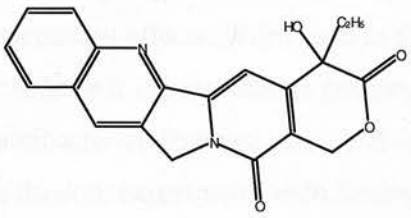


Figure 1.8. (*S*)-(+)-Camptothecin from *Camptotheca acuminate*.

1.3.3 Plant derived antibacterial and antifungal compounds

Unlike microorganisms, plants have proved a disappointing source of potent, selective antibacterial and antifungal agents (Newton et al., 2000). Whilst many species produce compounds with some degree of antibacterial or antifungal activity very few are both sufficiently active and non-toxic to be considered leads for the development of new antibiotics. For example, in a review of over 350 plants cited over the last 30 years as having antimycobacterial activity only 7 species yielded pure compounds with MIC values of 8μg/ml or less (Newton et al., 2000). Two examples of plant derived antibacterial compounds that have found clinical use are the alkaloid berberine and the simple phenolic eugenol.

1.3.3.1 Berberine from *Berberis* Spp.

Species of the genus *Berberis* (Berberidaceae) have been used traditionally in a variety of antimicrobial type roles and the alkaloid berberine present in *Berberis* Spp. has been administered for the treatment of bacillary dysentery (Rabbani et al., 1987) and has a wide range of antimicrobial activities. Although it readily intercalates with DNA and inserts into lipid bilayers it is relatively non-toxic and can be taken orally (Sun et al., 1988). Despite berberine's broad spectrum antibacterial activity it is not clear whether the therapeutic effect of the compound arises from its antibacterial activity or from secondary effects. When used in the treatment of enterotoxigenic *E. coli* and *Vibrio cholerae* it appears that its primary mode of action is antisecretory agent rather than antibacterial (Rabbani et al., 1987). Berberine also interferes with bacterial cell adhesion; experiments with *Streptococcus pyogenes* showed that at concentrations below its MIC, berberine caused an 8 fold increase in the release of the adhesin lipoteichoic acid from the cell surface (Sun et al., 1988) and that this could act to inhibit colonisation during the infection process. There is some interest in using berberine as a starting point for creating analogues with improved activity.

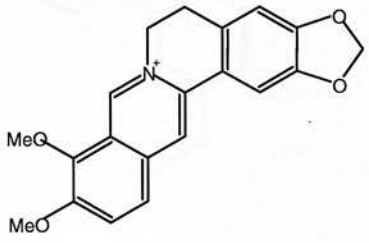


Figure 1.9. Berberine from *Berberis* Spp.

1.3.3.2 Eugenol

Eugenol is a simple phenolic derived from the clove tree *Eugenia caryophyllata* (Myrtaceae) which is indigenous to the spice islands of Indonesia, and Cloves have a long tradition of use as a treatment for toothache in both India and China (Curtis, 1990; Meeker and Linke, 1988). Reacting eugenol with zinc oxide produces zinc eugenolate chelate which has been used as a component of dental capping agents for many years due to its antibacterial and analgesic properties (Curtis, 1990; Meeker and Linke, 1988). Eugenol has broad spectrum antibacterial activity with a MIC of less than 1μg/ml for strains of *E. coli* and *S. aureus* and is effective at inhibiting the facultative anaerobes commonly isolated from infected root canals as well as certain moulds such as *Penicillium citrinium* (Walsh et al., 2003). The mode of action of eugenol remains unclear but experiments measuring ion flux suggest the bacterial membrane as a likely target. When susceptible strains of *E. coli* and *S. aureus* were exposed to eugenol rapid potassium loss indicative of membrane damage was observed (Walsh et al., 2003).

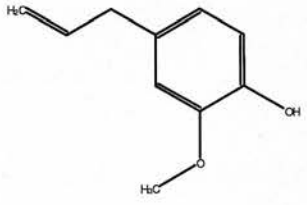


Figure 1.10 Eugenol from *Eugenia caryophyllata*.

Chapter 2

METHODS

2.1 Acquisition of plant material

Plant material for laboratory analysis was gathered through a process of ethnobotanical survey and "in the field" screening of plant material. For a more detailed account see the Chapter 3 (Fieldwork).

2.1.1 Gathering of ethnobotanical data and samples for screening

Information was gathered largely according to the "rapid ethnographic assessment" approach outlined by Etkin 1993 (Etkin, 1993). Three field work sites were selected on the basis of their differing ecologies and contacts were made in nearby villages with locals recommended by other villagers for their knowledge of plant medicines. Interviews were carried out in the local Neo Melanesian dialect with key informants to establish a range of candidate plants for screening, taking detailed notes on the use, preparation and local names of each species. Where possible, information was cross checked with secondary sources and where such information could not be corroborated it was discounted. An effort was made to focus on plants used to treat illnesses such as tropical ulcers that were likely to constitute some sort of microbial infection.

Once an exhaustive list of medicinal plants had been obtained, samples of each plant were collected for "in the field screening". This was done in conjunction with informants who had provided the source information. Samples were collected from the surrounding forest by informants themselves to prevent anomalies in the method of preparation occurring. Samples for initial screening were typically less than a gram of material and were collected into freezer bags labelled with the local name used for the plant.

2.1.2 In the field screening of plant material

Plants were screened in the field to avoid the need for a return to the fieldwork location at a later date. The field assay was based around the filter paper disk diffusion assay and the well diffusion assay in which samples are placed into pre-cut wells or disks in an agar plate. A strain of *Staphylococcus epidermidis* and *Escherichia coli* were stored as freeze-dried stocks in glass vials and opened when required. Bacteriological media was autoclaved in a miniature pressure vessel specifically designed for the purpose, (see Chapter 3 for more details) and bacterial cultures were grown in the ambient tropical temperature (approximately 30°C) and seeded onto sterile glass agar plates prepared with the pressure vessel. Ethanolic or aqueous plant extracts of each sample were made using a mortar and pestle and approximately 20µl or 5µl respectively were added to wells or disks on the plate and incubated over night. Active extracts were revealed by zones of inhibition of bacterial growth, small zones 1-2mm around the rim of the well were discounted.

2.1.3 Collection of botanical voucher specimens

Botanical voucher specimens were collected into 70% ethanol and sent for identification to the Papua New Guinea Forest Research Institute, Lae. Samples were prepared so as to show the overall morphology of the plant and its range of vegetative features. For smaller plants (shrubs) this meant collecting the whole plant including roots but for large tree species sections of branches showing the leaf arrangement along with sections of fruit and where possible flowers were all collected. Each voucher specimen was labelled with a number written in pencil on a jeweller's tag which was attached to each piece of the specimen. Each number was cross referenced to collection data which included a description of the location the plant was collected in, colours of fruit/flowers, the local name for the plant along with the ethnobotanical use and preparation. Specimens were placed into folded newsprint sheets and sealed in polythene before

adding alcohol. Where the water content of the plant material was high the water content of the alcohol was lowered accordingly. Export licences for voucher specimens were issued by the Papua New Guinea Department of Forestry after taxonomic identification had taken place.

2.1.4 Collection of insect specimens

A similar approach was taken for insect specimens recording location, local name and descriptions of use. Photographs were also taken to aid identification. Specimens were collected into 100% ethanol to reduce the likelihood of decomposition.

2.1.5 Collection of samples for laboratory analysis

After active species were identified with the field assay, bulk samples of more than 1kg were collected for lab testing. Where possible, specimens were collected from the original plant the initial screening sample was taken from. Specimens were dried in the shade on sheets of newspaper to avoid deactivation of chemical constituents by sunlight. For insect specimens the voucher specimen doubled as the sample for lab analysis.

2.2 Extraction of plant material

Plant material was extracted with a variety of different solvents to determine the optimum extraction conditions. For initial open column chromatography testing, bark was ground in a mortar and pestle whereas for later experiments the bark was ground in a coffee grinder and filtered in a Buchner funnel.

2.2.1 Small volume extracts for initial experiments

Extracts of each of the plants were made by grinding up 50mg of the dried plant material in the presence of 1ml of the solvent used (water or ethanol) with a mortar and pestle. The sediment was allowed to settle and the extract was filtered through Whatman No 1 paper. In some cases a cellulose acetate 0.2 µm disposable syringe filter was used to clarify the extract.

2.2.2 Large volume extracts for purification of active compounds

Larger volume extracts were made by grinding the bark in a coffee grinder to form a fine powder taking care to insure the raw material did not become over heated. The resulting powder was then transferred to a 500ml flask and 500ml of the solvent was added. The resulting solution was then stirred magnetically for at least 10 minutes to form a crude extract before being filtered through Whatman No 1 filter paper in a Buchner funnel using a water pump for suction. In some cases the crude extract was centrifuged at 3000 rpm for 1 minute to sediment out particles before filtration took place.

2.3 Concentration of fractions

Chromatography experiments tended to produce fractions more dilute than the sample mixture and so to maintain samples at a workable volume they were concentrated in a centrifugal evaporator, rotary evaporator or a lyophiliser (freeze-dryer).

2.3.1 GyroVap evaporator

A GyroVap evaporator connected to an Aquavac pump was used to concentrate chromatography fractions from so they could be easily assayed. The fractions were loaded into Ependorf tubes and these were spun at 1000rpm with the internal oven temperature set at 40°C.

2.3.2 Rotary evaporator

A Buchi Rotavapor rotary evaporator was used to concentrate samples where the sample volume was too large for the GyroVap. The rotary evaporator was connected to a water pump to provide negative pressure and was used in conjunction with a water bath set at 40°C.

2.3.3 Freeze-drying

Freeze-drying was carried out with an Edwards Muduylo system and used to concentrate aqueous samples where temperature and oxidation were a concern or before weighing pure compounds where traces of solvents would effect the accuracy of the reading. Samples containing small quantities of organic solvents were first treated in the rotary evaporator to remove traces of solvents that would prevent freezing. Samples were placed in a round-bottomed flask and frozen in a Cryobath CB-60 oil bath, spinning the flask by hand to ensure an even build up of ice on the inside of the flask. For small samples, (less than 1ml) the solution was frozen in an Eppendorf tube and a small hole pierced in the lid to allow equalisation of pressure. The Eppendorf tube was then placed in a round-bottomed flask that was connected to the freeze-dryer. In this way small volumes could be easily concentrated, weighed and then re-dissolved in small volumes of solvent.

2.4 Open column chromatography

A variety of chromatography separation media were trialled in open column format using gravity to feed the mobile phase through the column. In each case the separation media was swelled before pouring into a solvent resistant plastic column and allowing to bed out.

2.4.1 Gel filtration chromatography

Gel filtration was carried out using Pharmacia Sephadex LH-20 which was swelled in water for one hour allowing the fines to collect on the surface before decanting them. Water was replaced with the solvent of choice (normally ethanol) by filtering the Sephadex slurry through Whatman No 1 filter paper to remove water and then adding back the minimal amount of solvent required to produce a mixture sufficiently fluid to be poured into the column. The Sephadex was gently stirred so as to provide an even mixture which was then poured into a column held in a clamp at a 45° angle so the Sephadex ran down the sides of the column thereby displacing air bubbles. Once poured the column was allowed to stand upright to allow the Sephadex to form a bed before washing the column with several column volumes of solvent to clean it. Loading was achieved by running out excess solvent to a centimetre above the bed and then pipetting the sample into the column so that it ran down the side of the column and did not stir up the bedded Sephadex. The end cap and tubing were secured to the top of the column and the solvent reservoir placed high enough to produce the desired flow. The out flow line of the column was always connected to a Gilson FC 203B fraction collector and the drops/tube function used to programme it to collect 3ml or 5ml fractions.

2.4.2 Ion exchange chromatography

The cation exchange resin Whatman Microgranular CM52 or anion exchange resin Sigma Dowex-1 were first swelled with an equal volume of water for 10 minutes and then poured into a column using the same technique used for pouring Sephadex taking care not to introduce air bubbles into the resin. The resin was allowed to bed out before running the column first with a minimum of 10 column volumes of 1M pH 7.0 HCl Tris buffer and then a similar amount of 10mM pH 7.0 HCl Tris. Samples of the outflow were taken periodically and the pH taken to monitor the progress of the equilibration. Once the column was equilibrated the sample was loaded by pipetting the sample onto the inside surface of the column just above the resin bed, the end cap attached and the column run with the 10mM pH 7.0 HCl Tris solution for 10 fractions as a flow through to elute unbound material. The outflow line was connected to the fraction collector programmed with the drop/tube function to collect 3ml or 5ml fractions depending on the size of the loading sample. When elution proceeded very slowly an electrical pump was used to increase the flow rate.

After the flow through a salt gradient was then set up using a gradient maker, each chamber filled with 80ml 10mM Tris with varying amount of salt (NaCl, KCl or $MgCl_2$). The low salt chamber (connected to the inflow to the column) was stirred with a magnetic flea to insure constant mixing of the solutions and therefore a linear salt gradient but care was taken not to over stir as this caused a back pressure and uneven flow from the high salt to the low salt chamber. The salt gradient was normally run for sixty 5ml fractions and collected automatically as before. In some experiments a pH gradient was run by replacing the salt solutions for Tris buffer adjusted to different pH's.

2.4.3 Chromatography elution profile graphs

For each liquid chromatography experiment an elution profile graph was created of elution volume versus optical absorbance by taking spectrophotometer readings of each fraction at 280nm wavelength. Where small quantities of material were loaded onto columns, 1ml of each fraction was pipetted directly into a clean quartz quvette but where more concentrated extracts were used, fractions were diluted so as to keep the absorbance readings below 3.0. The dilution was carried out in an Eppendorf tube that was vortexed to ensure thorough mixing.

2.5 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) was carried out initially on an Applied Biosystems 130A machine and later on a Gilson system consisting of four 306 pumps and a 115 UV detector.

2.5.1 Set up and use of Applied Biosystems 130A and Gilson systems

For the applied Biosystems machine sample injection was carried out using a 100µl Hamilton Bonaduz Schweiz glass syringe with a 200µl loop fitted to the machine. Before injecting the sample the loop was cleaned by flushing through with 1ml distilled water. Flow rates ranging from 200 to 1000µl/min were programmed into the machine before starting each run. For flow rates above 500µl/min a gradient could not be sustained for long due to the low capacity of the solvent chambers in the machine. Instead, isocratic elutions were used where both chambers contained the same solvents so allowing the run to continue for longer without re-filling. This was achieved by selecting the free-run mode on the machine which allows changes in flow rate, % solvent B and duration of run to be altered during the course of the experiment.

At the end of each run the column was washed with a buffer rich in solvent B to elute any remaining material. Detection was set at UV 280nm in order to have the greatest chance of detecting material eluted from the column. This was recorded with a Kip and Zonen BD51 chart plotter run at 2mm/min with the variation set at 50mv. Samples were either collected manually as peaks appeared on the trace or over time using the fraction collector.

For the Gilson system different solvent gradients and flow rates of up to 1.5 ml/min were used. Injection was carried out automatically using a model 231 sample injector fitted with a 200µl loop. Integration was carried out by the Unipoint Software used to control the system.

2.5.2 Solvent preparation

Solvents were all HPLC grade unless stated and were filtered though Millipore 0.45µ filters to remove particles that would damage column packings if not removed. Filtering in this way also helped to degas solvents although in general the 75psi backpressure regulator fitted to the Gilson system prevented problems of out-gassing. Where solvent A alone contained salts care was taken to ensure that the salts would remain in solution at the full range of concentrations of solvent B during the gradient run. If when mixing the two solvents a precipitate was observed then a lower salt concentration was used.

2.5.3 HPLC experimental conditions

A wide variety of stationary and mobile phases were tried during the optimisation of the purification procedure. Listed below are the stationary phases, mobile phases and flow rates used. Unless stated otherwise the temperature was ambient. For each run numerous different gradient systems were tried.

2.5.3.1 Applied Biosystems Brownlee PTH C-18 5 micron 220 x 2.1mm reverse phase column

Flow rate 0.2ml/minute

Solvent A 0.1% TFA

Solvent B 0.08% TFA 70% MeCN

2.5.3.2 Supelcosil LC-Si 5 micron 250 x 4.6mm normal phase column

Flow rate 1ml/minute run isocratically

0.5% ammonia in methanol

0.1% TFA 70% MeCN

0.28% acetic acid 30% methanol

0.32% acetic acid 20% methanol

0.0425M pH 2.5 phosphate buffer 15% MeCN

0.003M pH 5.0 EDTA 70% Methanol

40% Methanol 0.05mM Heptane sulfonic acid adjusted to pH 3.5 with NaOH

70% Methanol 3mM pH 9.0 Tris HCl 0.09M KCl

70% MeCN 0.024% TFA 0.0015M Heptane sulfonic acid

2.5.3.3 Supelco supelcosil C-18 5 micron 250 x 4.6mm reverse phase column

Flow rate 1ml/minute run isocratically

0.28% acetic acid 9% methanol

80% methanol 20% 0.002M phosphate buffer pH 7.0

75% MeCN 1.25mM lauryl sulphate 0.0025% $HClO_4$

0.0425M pH 2.5 phosphate buffer 15% MeCN

0.007M EDTA pH 5.0 30% Methanol

0.003M pH 5.0 EDTA 70% Methanol

40% Methanol 0.05mM Heptane sulfonic acid adjusted to pH 3.5 with NaOH

60% Methanol 0.05mM Heptane sulfonic acid adjusted to pH 3.5 with NaOH

70% MeCN 0.024% TFA 0.0015M Heptane sulfonic acid

70% MeCN 0.024% TFA 3mM EDTA

Gradient runs

Solvent A 10mM pH 8 Tris 20% MeCN

Solvent B 100mM pH 8 Tris 20% MeCN 1.0M KCl

Solvent A 10mM pH 8 Tris HCl 1% Triethylamine

Solvent B 70% MeCN 10mM pH 8 Tris HCl 1% Triethylamine

Solvent A H_20

Solvent B MeCN

2.5.3.4 Supelcogel TPR 100 Polymeric column reverse phase column

Flow rate 0.5ml/minute

Solvent A 10mM pH 9 Tris HCl

Solvent B 10mM pH 9 Tris HCl 70% Methanol

Solvent A H_2O

Solvent B MeOH

Solvent A 10mM HCl KCl buffer pH 1
Solvent B MeOH

Solvent A 10mM Citrate pH 3
Solvent B MeOH

Solvent A 10mM Citrate pH 5
Solvent B MeOH

Solvent A Tris HCl pH 7
Solvent B MeOH

Solvent A Tris HCl pH 9
Solvent B MeOH

Solvent A 10mM Na_2CO_3/$NaHCO_3$ pH 10.6
Solvent B MeOH

2.5.3.5 Thermo hypersil ß-basic C-18 150 x 4.6mm reverse phase column

Flow rate 1.5ml/minute

Solvent A H_2O
Solvent B MeCN

Solvent A H_2O 5mM ß-mercaptoethanol
Solvent B MeCN ß-mercaptoethanol

Solvent A H_2O 5mM ß-mercaptoethanol
Solvent B methanol 5mM ß-mercaptoethanol

Solvent A H_2O 5mM ß-mercaptoethanol
Solvent B acetone 5mM ß-mercaptoethanol

Solvent A H_2O 5mM ß-mercaptoethanol
Solvent B tetrahydrofuran (THF) 5mM ß-mercaptoethanol

Solvent A 5mM heptane sulfonic acid
Solvent B 5mM heptane sulfonic acid 90% MeCN

Solvent A 10mM pH 9 phosphate buffer
Solvent B 10mM pH 9 phosphate buffer 70% MeOH

Solvent A 10mM pH 8 phosphate buffer
Solvent B 10mM pH 8 phosphate buffer 70% MeOH

Solvent A 0.1% Triethylamine 5mM ß-mercaptoethanol
Solvent B 0.1% Triethylamine 5mM ß-mercaptoethanol MeCN

Solvent A 0.2% ammonia 5mM ß-mercaptoethanol (pH 10)
Solvent B 0.2% ammonia 5mM ß-mercaptoethanol MeCN

Solvent A 10mM pH 8 Tris HCl
Solvent B 10mM pH 8 Tris HCl 70% MeCN

Solvent A 0.05M phosphate buffer pH 8
Solvent B 0.05M phosphate buffer pH 8 70% Methanol

Solvent A 0.1% phosphoric acid

Solvent B 0.1% phosphoric acid 70% methanol

Solvent A 0.2% phosphoric acid

Solvent B 0.2% phosphoric acid 70% methanol

Solvent A 300mM KCl

Solvent B MeOH

2.5.3.6 Thermohypersil Hypercarb 4.6 x 50mm normal/reverse phase column

Flow rate 1.5ml/minute

Initial gradient of 0 to 100% MeOH followed by 100% THF followed by a second gradient of 50% THF 0.05% TFA going to 100% THF.

Solvent A 0.1% TFA

Solvent B 1:1 Isopropanol/MeCN 0.1% TFA

Solvent A 0.1% TFA

Solvent B 1:1 Isopropanol/MeCN 0.1% TFA

Column temperature 70°C

Solvent A10mM KH_2PO_4

Solvent B Isopropanol

Solvent C MeCN

Solvent A 0.1% diethylamine

Solvent B 1:1 MeCN/IPA 0.1% DEA

2.5.3.7 Thermohypersil phenyl/hexyl betabasic150 x 4.6mm reverse phase column

Flow rate 1.5ml/minute

Solvent A 0.1% TFA

Solvent B 1:1 Isopropanol/MeCN 0.1% TFA

Solvent A 0.1% TFA

Solvent B 1:1 Isopropanol/MeCN 0.1% TFA

Column temperature 50°C

Solvent A 5mM ß-mercaptoethanol 0.1% TFA

Solvent B 5mM ß-mercaptoethanol 1:1 Isopropanol/MeCN 0.1% TFA

Solvent A H_2O

Solvent B 1:1 Isopropanol/MeCN

Solvent A H_2O

Solvent B 1:1 Isopropanol/MeCN

Solvent C 0.1% TFA

Solvent A 0.1% ethanolic acid

Solvent B 0.1% ethanolic acid 1:1 isopropanol/methylcyanide

Solvent A 0.1% phosphoric acid

Solvent B Methyl cyanide: isopropanol 1:1

Solvent A 10mM KH_2PO_4

Solvent B 1:1 MeCN/IP

Solvent C 0.1% TFA

Solvent A 50mM KH_2PO_4

Solvent B Isopropanol

Solvent C MeCN

Solvent D H_2O

Solvent A 100mM KH_2PO_4

Solvent B Isopropanol

Solvent C MeCN

Solvent D H_2O

Solvent A10mM HCl

Solvent B isopropanol

Solvent C MeCN

Solvent D H_2O

Solvent A10mM H_2SO_4

Solvent B isopropanol

Solvent C MeCN

Solvent D H_2O

Solvent A 0.1% Methanoic acid

Solvent B Isopropanol

Solvent C MeCN

Solvent A 0.5% Methanoic acid

Solvent B Isopropanol

Solvent C MeCN

2.6 Nuclear Magnetic Resonance Spectroscopy

The initial TFA lunacridine derivative was identified using both carbon-13 and proton NMR and allowed later samples to be identified using proton NMR alone. A variety of NMR techniques were used which are briefly outlined below.

2.6.1 Instrumentation

Nuclear magnetic resonance (NMR) spectroscopy was carried out by Dr Ian Sadler at the Ultra-High Field NMR Centre, Department of Chemistry, University of Edinburgh using a Varian INOVA 600MHz spectrophotometer.

2.6.2 Standard single pulse spectra

A short (microsecond) long radiofrequency pulse was applied to the sample and the resulting emission signal (Free Induction Decay - FID) collected. This signal was converted into a conventional spectrum by Fourier transformation used for a basic proton spectrum. A basic carbon-13 spectrum was similarly obtained except that broad-band proton decoupling was applied throughout the experiment to remove all proton couplings thus giving a single line for each carbon nucleus present.

2.6.3 Carbon-13 DEPT spectra

By a specially designed rapid sequence of pulses, rather than a single pulse it is possible to obtain selected parts of a spectrum only, thus simplifying the spectrum. The DEPT

sequence enabled us to obtain (a) a spectrum showing signals only from carbons bearing one hydrogen (b) a spectrum in which signals from CH and CH_3 carbons are positive and those from CH_2 carbons are negative.

2.6.4 HMQC Heteronuclear Multiple Quantum Coherence spectroscopy

This method was used to correlate proton resonances with those of directly bonded carbon-13 (or other) nuclei. The axes of the contour plot represent the proton and other nucleus chemical shift ranges. Signals occur at co-ordinates corresponding to the shifts of the bonded pairs of nuclei.

2.6.5 HMBC Heteronuclear Multiple Bond Correlation spectroscopy

HMBC is essentially the same as HMQC and was used for correlating proton and carbon-13 (or other) resonances showing mutual coupling through two or three bonds (occasionally more). This is usually optimised for couplings around 8 Hz and so only signals from those nuclei whose couplings lie in the 6-10 Hz range were likely to be observed.

2.7 Bacterial cultures

Cell cultures were made according to standard methods by inoculating autoclaved broth with cells from a single colony removed from a plate on which the stock culture had been streaked out. For assaying semi pure fractions, cultures grown in LB broth were used and these were made by autoclaving 2.5g of LB powder (Oxoid) in 100ml water. For *S. aureus*, m-*Staphylococcus* broth was used which contained the following ingredients:

- dH_2O 100ml
- Agar 1.5g
- NaCl 7.5g
- Mannitol 10g
- Tryptone 10g
- K_2HPO_4 0.5g
- Yeast Extract 0.25g
- Lactose 0.2g

For MIC experiments Isosensitest agar was used and made up according to the manufacturers instructions, (11.7g isosensitest broth (Oxoid) autoclaved in 0.5L distilled water).

Once inoculated the nutrient broth was incubated at 37C° overnight to produce log phase cells. Strains of bacteria used included *E. coli (E. coli)* ATCC 10536 and *S. aureus* NCTC 6571 as well as NCTC 12493 (methicilin resistant *S. aureus*). Agar plates for assaying semi-pure fractions were made by autoclaving LB medium containing 25g LB powder, 15g bacteriological agar and 1 litre de-ionised water in a 1 litre Gibco bottle, allowing to cool until hand hot before pouring into 14.5cm x 2.1cm sized plates. Plates were generally poured to a thickness of 1.2cm for the well assay and 0.7cm for the paper disk assay. For MIC experiments 15.7g isosensitest agar and 11.7g isosensitest broth (Oxoid) were autoclaved in 0.5L distilled water and 20ml poured into plates. All autoclaving was run on a cycle at 15 pounds per square inch for 20 minutes.

2.8 Bacterial inhibition assays

To assay fractions the well method or Kirby/Bauer filter paper disk diffusion assay were used depending on the activity of the sample. More quantitative information was obtained using the agar and broth dilution assay.

2.8.1 Well method

Plates were poured to a thickness of approximately 1.2cm and allowed to harden. The agar was then seeded with the appropriate cells by pouring 5 to 10ml of the broth cell culture directly on to the agar making sure to cover the whole plate, excess culture was then removed with a sterile pipette. A glass pipette with an open end 6mm in diameter was then sterilised by flaming it with ethanol. Once cool, the sterile pipette was used to cut holes in the agar, (one for each sample to be tested). The resulting plugs of agar were then removed with sharp tweezers which were also sterilised by flaming with ethanol. The wells were injected with samples ranging in volumes from 10 to 100µl depending on the size of the sample being assayed. One of the wells was always loaded with the solvent the samples were prepared with to act as a negative control. The plate was then placed in an incubator overnight at 37°C. Zones of inhibition surrounding active samples were measured by their diameter from underneath the plate. Solvent controls rarely produced significant zones of inhibition.

2.8.2 Filter Paper disk method

This assay was used where samples had high enough activity to show up with small volumes. Plates were poured to a thickness of approximately 0.7cm, allowed to solidify before being inoculated with cell culture as above. Excess cell culture was removed and

the plates left to dry with the lids partially removed. Filter paper disks 6mm in diameter were made by cutting holes in blotting paper with a hole punch. Autoclaved disks were impregnated with the test samples by pipetting 3-5µl of sample onto each disk. The disks were then placed onto the agar with sterile tweezers and their positions recorded with marker pen on the lid of the dish. As with the well assay the plate was then placed in an incubator overnight at 37°C and zones of inhibition surrounding active samples were measured by their diameter from underneath the plate.

2.8.3 MIC by agar dilution

MIC experiments were carried out using the agar dilution method according to the protocol stipulated by the Journal of Antimicrobial Chemotherapy (2001) (Andrews, 2001). Drugs were added to 20ml molten agar from stocks of specified concentrations (10, 1 and 0.1mg/ml). For example, for 128µg/ml 256µl of a 10mg/ml stock were used or for 1µg/ml 200µl of a 0.1mg/ml stock were used. In each case the drug volumes were aliquoted into sterile autoclaved flasks and 20ml hand hot molten agar (approximately 50°C) made from 15.7g isosensitest agar and 11.7g isosensitest broth (Oxoid) autoclaved in 0.5L distilled water.

For each concentration the molten agar was added to the flask, mixed by swirling once before pouring directly into 90mm Petri dishes. Molten agar was added to each drug concentration in turn and poured straight into the Petri dish so as to minimize the amount of time the drugs were exposed to 50°C temperatures. The plates were allowed to cool and once solidified were dried for 10 minutes in an incubator with the lids partially removed resting on the rims of the plate. An overnight culture of cells was prepared as described above and adjusted to the same opacity as a 0.5 McFarland standard (purchased from BioMeriux Basingstoke UK) with sterile isosensitest broth and then diluted ten fold in sterile distilled water. 1µl of the diluted culture was added to the surface of each plate and allowed to stand with the lid partially removed in the incubator

until the inoculum had been visibly seen to be absorbed into the agar. A no inoculum control was generally included to check for contamination as well as a no drug control to ensure the cell culture was viable. The plates were incubated for 18 hours at 37°C and then inspected visually for signs of growth. The MIC was defined as the lowest concentration of drug at which there was no visible growth of the organism. The presence of one or two colonies or a thin film of growth was disregarded as stipulated by the Journal of Antimicrobial Chemotherapy (2001) method (Andrews, 2001).

2.8.4 MIC by broth microdilution

The broth microdilution method described by Andrews et al (Andrews, 2001) was used for isolated plant compounds since the amounts of material available were insufficient to be used with the agar dilution method. Briefly, 75µl of each drug concentration were added to a sterile Nunclon (Nunc) 96 well plate using the same antibiotic solutions as for the agar dilution method. The bacterial cultures were prepared as described above and adjusted to the same opacity as the 0.5 McFarland standard before diluting 100 fold with isosensitest broth to produce a final concentration of 10^5 CFU/ml. 75ul of diluted broth was then added to each well and the plate incubated for 18 hours. On each plate a no drug control and uninoculated well was included to allow comparison of both full and no growth. The MIC was defined as the lowest drug concentration in which there was no visible growth.

2..8.5 *Streptococcus pneumoniae* disk diffusion assay

The *S. pneumoniae* disk diffusion assay was carried out by Professor S. Amyes (Department of Medical Microbiology, University of Edinburgh). Lunacridine impregnated disks were screened against *S. pneumonia* strains using the Kirby/ Bauer filter paper disk diffusion assay. Bacterial cultures were

prepared according to NCCLS guidelines and seeded onto Mueller-Hinton agar plates supplemented with 5% defibrinated horse blood. Sterile filter paper disks 6mm in diameter each containing 200μg TFA lunacridine were added to plates inoculated with each strain. The plates were cultured for 24 hours at 37°C in 5% CO_2 and then inspected for zones of inhibition which were measured from the front of the plate.

2.9 HSV-1 virus yield reduction assay

The assay required specialised lab conditions and so was carried out by Dr M. Ogilvie, at the specialist virology centre at the Edinburgh Royal Infirmary

2.9.1 Cell culture

Briefly, the virus yield reduction assay was carried out using a known HSV-1 reference strain SC16 using the nucleoside analogue acyclovir as a positive control. SC16 was grown on confluent monolayers of both MRC-5 fibroblasts and African green monkey kidney (Vero) cells which were cultured as follows:

2.9.1.1 MRC-5 cell line

10% minimal essential medium (MEM) supplemented with 10% heat inactivated foetal calf serum, 1% sodium bicarbonate (7.5%), 1ml L-glutamine and 1ml penicillin and streptomycin (100 units/ml penicillin, 100μg/ml streptomycin). Maintenance medium containing 2% foetal calf serum was added once cell were confluent.

2.9.1.2 Vero cells

10% minimal essential medium (MEM) supplemented with 2% heat inactivated foetal calf serum, 1.5% sodium bicarbonate (7.5%), 1ml L-glutamine and 1ml penicillin and streptomycin (100 units/ml penicillin, 100µg/ml streptomycin). Maintenance medium containing 1% foetal calf serum and 2.4% sodium bicarbonate (7.5%) was added once cells became confluent.

2.9.1.3 HSV-1 SC16

HSV-1 SC16 from stocks was added to a 75cm^2 flask of confluent MRC-5 cells and once 100% infected the contents were centrifuged briefly and the supernatant removed and stored as frozen 2.5ml aliquots at -20°C.

2.9.2 Yield reduction assay

MRC-5 cells or Vero cells were seeded into a 24 well microtitre plate at 2 x10^5 and 1 x10^5 cells/ml respectively. Once confluent, the culture was inoculated with 200µl HSV-1 SC16 supernatant at a multiplicity of infection of 5 plaque forming units per cell. The plate was then incubated at 36°C for 1 hour to allow virus adsorption whereupon the inoculum was replaced with 1ml aliquots of TFA lunacridine or acyclovir at varying drug concentrations.

The plate was incubated at 36°C overnight after which the cells were lysed by freeze-thaw at -80°C. 100µl aliquots were then taken from the infected wells and transferred to a fresh plate and serially diluted 1:3 across the next five columns of the plate. The plates were incubated at 36°C for one hour to permit virus adsorption whereupon virus inoculum (containing test compounds) was replaced with 0.2ml fresh medium. The plates were then incubated for a further 2 days after which medium was removed, and

the cell sheets fixed with methanol. The medium was then removed from each well and the cells stained with Wrights stain for 10 minutes, rinsed with PBS and allowed to dry. Viral plaques were counted visually.

2.10 Human cell cytotoxicity assays

Cytotoxicity assays were performed using the Cell Titer 96 Aqueous One solution cell proliferation reagent (Promega) which contains the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS[(a)]] and an electron coupling reagent (phenazine ethosulfate; PES). Reduction of the reagent as a result of cellular metabolic activity results in the production of a coloured formazan product whose absorption at 490nm is directly proportional to the number of living cells.

MRC-5, H226 and HELA cells were grown in tissue culture flasks in a Sanyo CO_2 incubator set at 37°C and 5% CO_2, plated into 384 well tissue culture plates and allowed to attach overnight before adding drugs and then reagents. Throughout the assay sterile techniques were observed and all liquid handling and cell culture was carried out in a class II microbiological safety cabinet which was swabbed down with 70% IMS before use. Care was taken to avoid contact between skin and lab materials and latex gloves were worn at all times. Pipetting of liquids was done using an electric pipettor (Finnpipette) using sterile 10ml disposable pipette tips. Cells suspensions were added to plates using a 16-channel pipette (Finnpipette).

2.10.1 Cell culture

MRC-5, H226 and HELA cells were obtained as frozen stocks maintained in liquid nitrogen and were rapidly thawed out by placing in a water bath at 37°C before adding to 2-3ml pre-warmed culture medium in a 75cm^2 tissue culture treated flask with a filter

cap lid (filter cap lids were used with all tissue cultures to reduce the risk of contamination). The cells were allowed to grow to near confluency before splitting and transferring to a larger 175 cm^2 flask. Splitting of cells was carried out for a 175cm flask as follows, and varied according to the size of the flask.

Cell culture medium was removed from the culture flask which was then washed out with phosphate buffered saline (PBS) to remove traces of culture medium which would inhibit the trypsin solution. 1X Trypsin solution (Gibco) was then added in sufficient quantity to just cover the surface of the culture flask but no more and swirled slightly to ensure it was evenly distributed. The flask was then placed back in the incubator where it was left for 2-3 minutes to allow cells to detach after which the flask was removed from the incubator and tapped on each side several times to help fully detach cells. To achieve an even suspension the detached cells were pipetted up and down ten times, each time allowing the cells to run down the bottom of the flask. Between a half to two thirds of the detached cells were discarded (depending on the cell type) and 15ml culture medium was added back to the flask, which was then returned to the incubator. Media for each of the cell lines was as follows:

2.10.1.1 MRC-5 cells

- 500ml minimal essential medium (MEM) + Earle's – L-glutamine (Gibco)
- 50ml foetal bovine serum (heat inactivated) (Gibco)
- 5ml L-glutamine pen/strep solution (200 mM L-glutamine10,000 units/ml penicillin, and 10 mg/ml streptomycin in 0.9 % sodium chloride) (Sigma).
- 5ml MEM 100x non-essential amino acids (Gibco)
- 5ml 100 mM Na Pyruvate (Sigma)

2.10.1.2 H226 cells

- 500ml RPMI 1640– L-glutamine (Gibco)
- 50ml foetal bovine serum (heat inactivated) (Gibco)
- 5ml L-glutamine pen/strep solution (200 mM L-glutamine10,000 units/ml penicillin, and 10 mg/ml streptomycin in 0.9 % sodium chloride) (Sigma).
- 5ml 100 mM Na Pyruvate (Sigma)

2.10.1.2 HELA cells

- 500ml minimal essential medium (MEM) + Earle's – L-glutamine (Gibco)
- 50ml foetal bovine serum (heat inactivated) (Gibco)
- 5ml L-glutamine pen/strep solution (200 mM L-glutamine10,000 units/ml penicillin, and 10 mg/ml streptomycin in 0.9 % sodium chloride) (Sigma).
- 5ml 100 mM Na Pyruvate (Sigma)

To harvest the cells for the assay the same procedure outlined for splitting the cells was followed. Cells were split before they approached confluency and culture media changed 24 hours prior to splitting to ensure cells were log phase.

2.10.2 Plating out of cells

After trypsinisation of the cells with 3ml pre-warmed trypsin solution, a 20µl aliquot of the cell suspension was removed and placed adjacent to the cover slip of a $0.1mm^3$ haemocytometer and the number of cells counted and converted into a per ml figure. The trypsinised cells were then diluted to 2.5×10^5 cells per ml with pre-warmed culture medium and pipetted up and down twenty times to ensure any cell clumps had been removed since a single cell clump would greatly increase the number of cells in a given well and so result in an erroneously high reading of cell viability. 25µl aliquots of the resulting cell culture were then added to sterile transparent optical 384 well plates (Nunc) in columns of 8 or 16 wells at a time using the 16 channel Finnpipette to produce a final count of 6,250 cells/well in accordance with Nuncs recommendation of 5000 to

20 000 cells per well in 384 well plates. Care was taken to ensure the culture was delivered to the bottom of each well and all the tips of the pipette were attached securely. Plates were then placed in the incubator overnight for the cells to attach.

2.10.3 Incubation with drugs and Cell Titer reagent

Drug solutions were made up by diluting 5mg/ml aqueous drug solutions with the appropriate cell culture medium to make 200μM solutions. A range of concentrations were achieved by serial dilution along the columns of wells removing and discarding 25μl of media from the penultimate column. The last column was left as a no drug control so that each cell line/drug had its own no drug control. Further control wells of 25μl uninoculated culture medium were included to control for background absorbance. Plates were incubated for 4, 12, 24, 48 or 72 hours after which 5μl of the Cell Titer dye compound (pre-warmed to 37°C) was added to each well including all the control wells and the plate incubated for a further 2 hours. The plate was removed from the incubator and 5μl isopropanol added to each well to remove air bubbles which would affect the reading. The plate was then read in a Wallac Victor 1420 multi label counter which was set to shake the plate for 1minute before reading the optical absorbance of each well at 490nm. Controls of the drugs at their highest concentrations were also included but were found not to absorb at 490nm. Graphs were then plotted with the cell viability expressed as % of the no drug control subtracting background absorbance.

2.11 Caspase-3/7 assay

The caspase-3/7 assay was carried out as for the cell viability assay with minor modifications and replacing the Cell Titer reagent with the Apo-1 caspase-3/7 reagent (Promega) using white non-transparent fluorescence plates. The Apo-1 reagent consists of a lysis buffer which allows release of cellular caspases and a profluorescent caspase-

3/7 consensus substrate, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide) rhodamine 110 (Z-DEVD-R110). Upon cleavage on the C-terminal side of the aspartate residue in the DEVD peptide substrate sequence by caspases-3 or 7, the rhodamine 110 is free to fluoresce at 521nm when exited at 498nm. The reagent was made up according to manufacturer's instructions by diluting the rhodamine substrate 1:100 with the lysis buffer and warmed to 37C° in a water bath.

After addition of the Apo-1 reagent the plates were shaken using the Wallac Victor 1420 multi label counter and then incubated for 7 hours on a bench top with the lid on before reading fluorimetrically at 498nm excitation and 521nm emission using the same Wallac Victor 1420 multi label counter. No drug controls were included on each plate along with wells containing each drug at its highest used concentration which was not found to have an effect on fluorescence. Graphs were then plotted as % increase in fluorescence over the no drug controls.

2.12 DNA intercalation assays

DNA intercalation assays were carried out according to the method described by Cain et al (Cain et al., 1978). SHE buffer containing the following was made up accordingly.

- 2mM Hepes
- 10μM EDTA
- 9.4mM NaCl
- 1.26μM ethidium bromide

The pH was then adjusted to 7.0 with NaOH as described. To carry out the assay, 3ml of the SHE buffer was placed in a plastic cuvette with the corrugated side facing the excitation beam of a Hitachi F-2000 fluorescence spectrophotometer. Excitation wavelength was set at 546nm and emission at 595nm. 2μl of 1 mg/ml solution of calf

thymus DNA (Invitrogen) was then added to the SHE buffer and stirred vigorously with a blue tip to ensure thorough mixing. Drugs were then added in a stepwise fashion and readings taken making sure that the contents of the cuvette were properly mixed after each drug addition.

2.13 Reverse transcriptase assays

Reverse transcriptase assays were carried out using the reverse transcriptase assay colorimetric kit (Roche Diagnostics) in which HIV-1 reverse transcriptase activity is detected with an ELISA type protocol.

Digoxigenin and biotin-labelled nucleotides are included in the kits reaction mixture in an optimised ratio and once incorporated into the DNA reaction product they allow binding of the DNA to the surface of the streptavidin-coated microplate wells the reaction is carried out in. This allows unincorporated labelled nucleotides to be washed away so that in the next step which involves addition of an anti-digoxigenin peroxidase antibody conjugate (anti-DIG-POD), only synthesised DNA is bound by the antibody. In the final step, the peroxidase substrate ABTS is added and converted to a coloured reaction product thereby giving a quantitative reading of DNA reaction product and therefore RT activity.

2.13.1 Procedure

To carry out the assay drugs were first dissolved in lysis buffer (50mM Tris, 80mM KCl, 2.5mM DTT, 0.75mM EDTA and 0.5% Triton X-100 pH 7.8). Next 20µl aliquots of reconstituted enzyme (2ng/µl corresponding to 10 mU/µl) were placed in sterile reaction tubes and 20µl aliquots of the drugs at the desired concentrations were added. A further

20µl of the reaction mixture (46mM Tris-HCl, 266mM KCl, 27.5mM $MgCl_2$, 9.2mM DDT, 10µM dUTP/dTTP, template/ primer hybrid, 750m$A_{260\ nm}$/ml) were added last.

The tubes were then incubated at 37°C for one hour. Controls of lysis buffer only and lysis buffer with the highest concentration of each inhibitor were included. Each of the 60µl reaction samples were transferred to the streptavidin-coated microplate wells and the whole 96 well plate covered with a plastic film to prevent evaporation and incubated for 1 hour at 37°C. The solution was then removed from each well and washed five times with 250µl washing buffer (supplied with the kit) allowing the washing buffer to sit in each well for at least 30 seconds before removal.

200µl of anti-digoxigenin-peroxidase polyclonal solution (200mU/ml) were added to each well, covered as before and incubated for 1 hour at 37°C. The antibody solution was removed by inverting the microtitre plate before rinsing each well with washing buffer as before. 200µl ABTS solution (supplied with kit) were then added to each well and incubated at room temperature for 20 minutes after which time a dark green colour developed in the no-drug control and other wells with low RT activity. The absorbance of each well was then read at 405nm (reference wavelength 490nm) using a Wallac Victor 1420 multi label counter. Graphs of RT activity were plotted as % of the no drug control subtracting background absorbance. None of the drugs used in the experiment were found to absorb at 405nm at the highest concentrations used.

2.14 Topoisomerase II decatenation assays

Topoisomerase assays were carried out using a kit supplied by Topogen Inc. The kit used a kinetoplast (kDNA) substrate which when decatenated by topoisomerase II alpha (supplied with the kit) displayed a clear increase in mobility when run on an agarose gel. The large size of the kDNA relative to its monomeric reaction product ensured a significant difference in mobility between the two.

Reaction mixtures generally contained in order of addition; sterile water to 20µl, 2µl 10X reaction buffer (50mM Tris-HCL pH 8, 120mM KCl, 10mM $MgCl_2$, 0.5mM ATP, 0.5mM DTT and 30µg BSA/ml),1µl kDNA substrate, drugs in dH_2O and 1µl topoisomerase II enzyme supplied with the kit. The reactants were always added in that order to prevent initiation of the reaction before all the reactants were added or denaturation of the enzyme due to addition of undiluted reaction buffer. The final volume of each reaction was 20µl and the reaction mixture was incubated for 1 hour at 37°C after which the reaction was halted by addition of one fifth volume of stop buffer loading/dye supplied with the kit.

The contents of each reaction tube were then run on a 1% agarose gel at 90V using TAE as running buffer(40mM Tris buffer 20mM glacial acetic acid 1mM EDTA). Gels were made by heating 200mg of electrophoresis grade agarose with 20ml TAE buffer in a microwave until the resulting solution began to boil. A lid was used when preparing the gel mixture to prevent evaporation changing the gel concentration. Once cool enough to handle, the molten gel was poured into its mould and allowed to harden before being placed inside a gel tank and filled with TAE running buffer. In each experiment a positive (no drug control) was included and in the earlier experiments a marker of decatenated kDNA to indicate the position of reaction product. A linear kDNA marker was also used to check for degraded kDNA substrate. DNA was visualised by staining the gel in 50ml of a 1µg/ml solution of ethidium bromide, rocking gently for 10 minutes and then briefly de-stained in water to reduce background fluorescence.

Chapter 3

ETHNOBOTANICAL FIELDWORK

3.1 The ethnobotanical approach

Conventional ethnopharmacological studies are multidisciplinary and usually require several participants from different fields (Malone, 1983). There is normally an ethnobotanist who provides candidate species for screening based on their use in folk medicines, a chemist to isolate and identify the structures of chemical constituents (Malone, 1983) and in the case of antimicrobial plants a microbiologist to assay fractions and gather data on the activity of purified compounds. Plants samples are normally collected by the ethnobotanist and sent back to a laboratory where testing begins; about a kilogram of material is generally required to allow purification of active compounds without having to return to the field to re-supply (Macilwain, 1998). This presents a problem in that such large quantities of plants are difficult to transport and collecting them would be inefficient since only a small number could be expected to posses any sort of antimicrobial activity.

To increase the chances of finding active plant species it was decided to develop an antibacterial field assay kit which would allow detection of plants with antibacterial activity in the field. Such an approach would allow one to assay fresh plant preparations before activity is lost during transportation. It would also eliminate the need to export large quantities of plant material which based on the premise that at least a kilogram of material is needed to give a reasonable chance of isolating mg quantities of pure compounds could be expected to run into hundreds of kilograms.

3.2 The development of the antibacterial assay kit

It was reasoned that a field kit based on the paper disc diffusion assay could be simple enough to allow reliable screening of plant extracts in the field provided methods for sterilising glassware and maintaining viable bacterial cultures could be established. To this end a lightweight pressure vessel was made that would allow glassware and culture

media to be heated above 100°C for sterilisation. A small stainless steal container was fitted with a rubber pressure seal and a pressure release valve and a methanol burner used as the heat source. After safety testing to ensure the pressure release valve functioned properly the system was tested by heating a culture of *Staphylococcus aureus* mixed with soil for 15 minutes. The culture was subsequently plated out and incubated overnight before inspecting for colonies. The plate was devoid of signs of microbial growth indicating complete sterilisation of the culture and soil sample.

The second part of the problem was to find a way of producing bacterial cultures in the field. It was speculated that freeze-dried samples of a gram positive and gram negative species could be kept at ambient temperature over the course of the field work period and revived in culture medium when required. Furthermore there would be no need for an incubator since the daily temperatures in a tropical rainforest range from 20°C to 30°C. The kit contained the following items.

- Light weight pressure vessel.
- Alcohol burner (Trangia).
- Glassware (Petri dishes, bottles etc).
- Freeze-dried cultures of *S. epidermidis* and *E. coli* (Selectrol).
- LB media as powder (Oxoid).
- Agar as powder (Oxoid).

The kit was highly portable and lightweight, and so ideally suited to be taken to the remote locations selected as fieldwork sites (Fig. 3.1). To produce bacterial cultures, a Selectrol disk containing freeze-dried bacteria (*S. epidermidis* and *E. coli*) was placed into sterile LB broth and incubated overnight or until a the culture medium became translucent. The agar and LB media were kept in numerous screw cap vials with silica gel desiccant to reduce exposure to humidity.

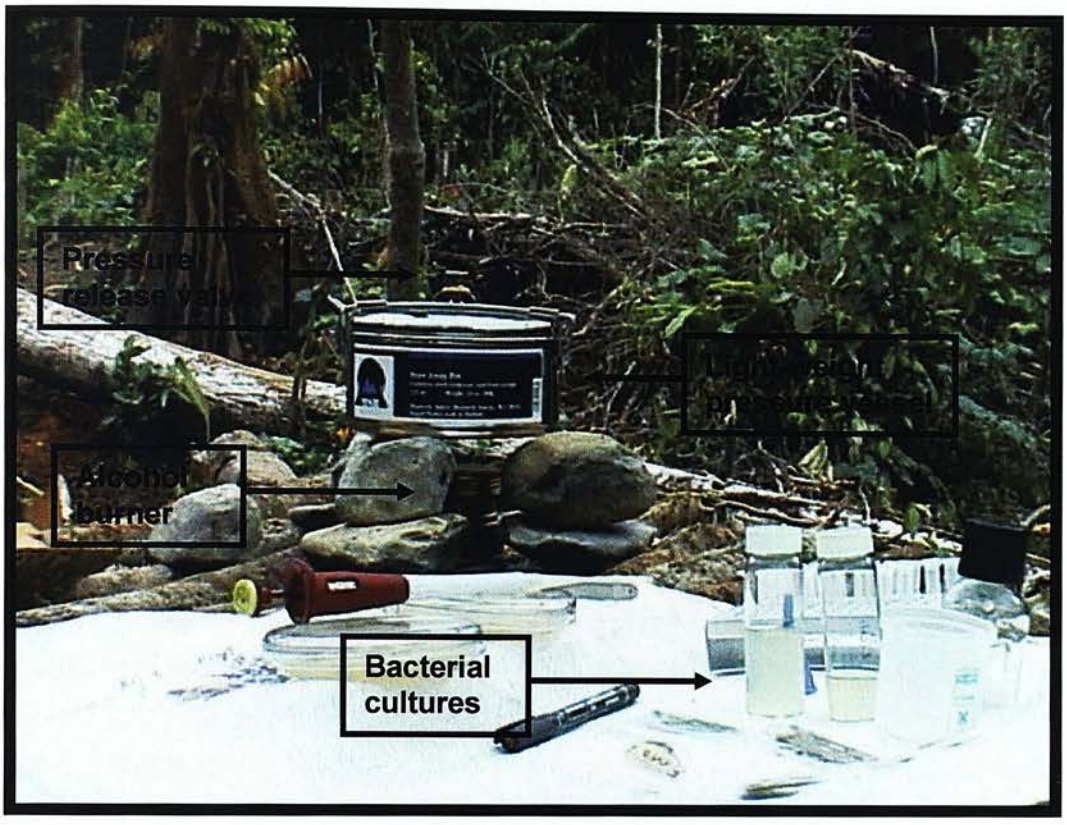


Figure 3.1 The field assay kit in use in the Whiteman range, Papua New Guinea.

To assay plants, aqueous and ethanolic extracts were made by grinding up plant material in a mortar and pestle in the presence of solvent. Where possible the preparation methods described in the ethnobotanical survey such as the addition of slaked lime ($CaOH_2$) made from burning local limestone deposits were used. Extracts were then applied to paper disks or wells cut into a Petri dish and left overnight at ambient temperature (typically 20-30°C) after which the plate was examined for zones of inhibition. Control wells or disks containing extraction solvents were used as controls. During the course of the fieldwork it was found that adding the extracts to wells cut into the agar rather than to paper disks allowed for greater sensitivity in detecting antibacterial activity.

3.3 Selection of field work sites

New Guinea has been described a biodiversity "hotspot" since it contains one of the largest undisturbed areas of rainforest in the world and at least 6,500 species of plants found nowhere else on earth. An estimated two-thirds to three-quarters of the land area is highly species diverse undisturbed tropical moist forest containing some 11,000 plant species, of which as many as 90 percent may be endemic (Myers, 1988). In addition to this New Guinea possesses some of the longest human inhabited rainforest in the world so providing the greatest possible length of time for local populations to develop a knowledge of medicinal plants through trial and error. Excavations carried out in the Whiteman Range of New Britain have provided evidence of inhabitation dating back over 35 000 years (Pavlides, 1994).

A wide variety forest types are represented ranging from the mangroves and swamp forests through to upper montane forests which in some cases continue up to 4000m above sea level (Whitmore, 1984). The ecological diversity is matched by ethnographic and linguistic diversity; estimates for distinct ethno-linguistic groups within Papua New Guinea range from 700 to 850 (Wurm, 1981). Since each group has their own knowledge of medicinal plants which differ according to ecological habitat it is not surprising that an estimated 1,035 different plant species are known to be used for specific purposes e.g. medicinal, agricultural, structural, decorative etc (Powell, 1982).

3.3.1 Location of fieldwork sites

With this is mind three fieldwork locations were selected on the island of New Britain covering the entire altitudinal range of inhabited rainforest on the island (Fig. 3.2). These were as follows:

1. The tip of the Williamez Peninsula (Bulu ethnolinguistic group).
2. The foothills of the Whiteman Range (inland Kaulong ethnolinguistic group)
3. The Nakanai Mountains (Mamusi ethnolinguistic group)

Each location represented a distinct population group and different rainforest type so as to minimise the likely overlap in plant species revealed by any subsequent ethnobotanical survey.

New Britain is Papua New Guinea's largest offshore island comprising 36,520 sq km of land area, almost all of which is forest covered; the climate is described as tropical-wet receiving between 3000mm to 6000mm of rain per annum (Lamoreux, 2001). The island was never attached to mainland New Guinea but instead emerged from the ocean floor as a result of volcanic activity during the late Miocene period (8-10 million years ago) and as a result most of the soil is either acidic volcanic or limestone (Mueller-Dombois, 1998).

The forest is predominantly lowland in type but the backbone of the island is punctuated by several mountain ranges providing extensive areas of terrain between 1000m and 2000m isolated from each other by lowland forest (Lamoreux, 2001). The boundary between lowland and montane forest is set at 1000m at which point the forest height, leaf and crown size diminishes, the temperature drops and the humidity increases (Mueller-Dombois, 1998). There are currently no comprehensive modern botanical datasets for New Britain, and much of the area is unknown in terms of biodiversity for any taxa (Sekhran, 1994).

In each fieldwork location, several informants were independently interviewed in the local Neo Melanesian dialect about the uses of medicinal plants and an extensive list of local plant names, uses and mode of preparation were recorded. Voucher specimens of each plant were collected and sent to the Papua New Guinea Forest Research Institute

for taxonomic identification. In many cases specimens were not fertile and so identification to the species level was not possible. Care was taken to only include plants whose uses could be independently verified by at least two separate sources. In the Bulu language group area (Williamez Peninsula) this was done by carrying out the study in two separate villages but in the Whiteman Range and Nakanai Mountains this was not possible.

A limitation of the ethnobotanical methodology was the lack of first hand evidence for the use of individual species. The number of times the author was able to observe plants being prepared and used to treat specific ailments was very limited indeed. This may be due in part to the rarity with which plants are used or to the increasing availability of commercially produced medicines available in towns on the edge of the forest. Some informants described the use of amoxicillin capsules which could be broken open and the contents applied directly to tropical ulcers so as to avoid the expense of a full course of treatment.

Despite this, in each location the respondents were able to name and describe a wide variety of species used for a range of medicinal purposes, many of these consistent with an antibacterial type role. These included the treatment of infected cuts, eye infections similar to conjunctivitis, dysentery, respiratory infections and tropical ulcers. Amongst the coastal populations (Williamez Peninsula) dysentery was the most common microbial-associated use for plants whereas in the Whiteman and Nakanai mountain ranges, tropical ulcers represented the most frequent condition plants were used to treat.

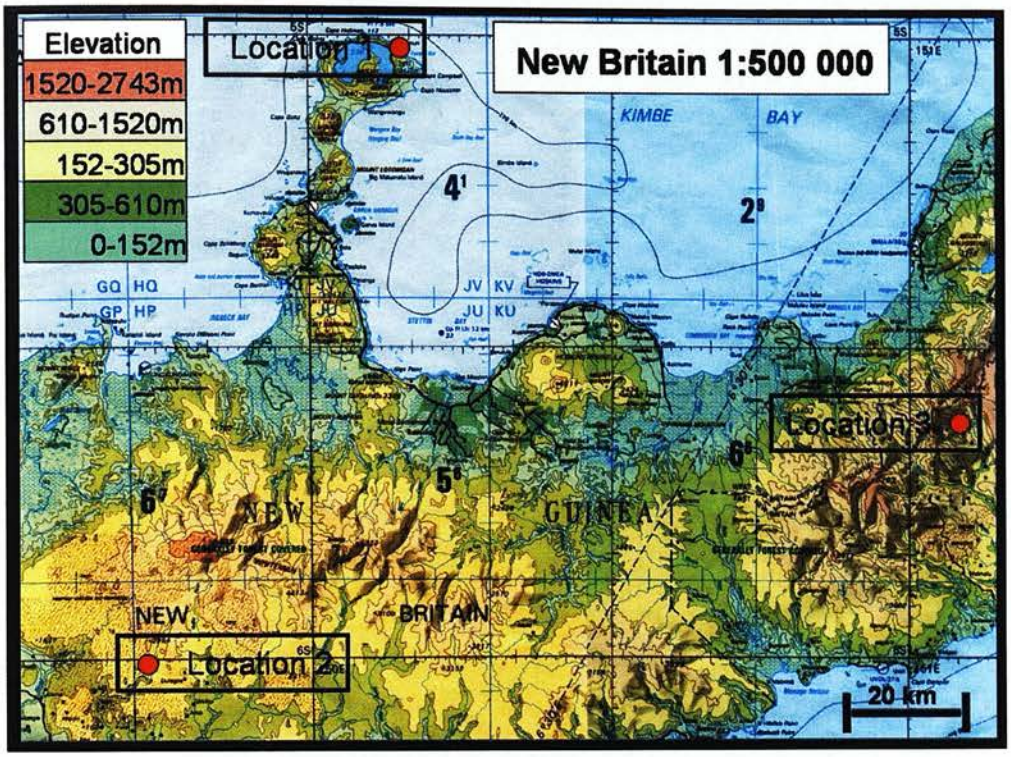


Figure 3.2 Location of field work sites on the island of New Britain, Papua New Guinea. Adapted from tactical pilotage chart M-14D produced by the RAAF, Victoria Barracks, Melbourne, Australia.

3.3.1.1 Location 1. The tip of the Williamez Peninsula

The Williamez peninsula is a remarkable physiographic feature, (Swartzendruber, 1993) and includes a very diverse area of lowland rain-forest on rich volcanic soils (Swartzendruber, 1993). The forest is almost entirely lowland although there are several volcanoes approximately 1000m in height. The climate is monsoonal giving rise to a distinct dry season. The local population forms a distinct ethno-linguistic group called the Bulu and number approximately 600 (Johnston, 1980). Their villages and hamlets are distributed exclusively along the coastal shoreline and in the centre of the peninsular is a large caldera lake but it supports little aquatic life.

Amongst the plants used by the villagers was *Derris alata* whose roots were employed as a fish stun poison. The roots are first pounded to release the sap and then packed into crevices in the surrounding coral reef. This caused the fish to become paralysed so allowing them to be easily taken from the water. *Derris* spp. are known to contain rotenoids that bind NADH:ubiquinone reductase (complex I) of the respiratory electron transport chain (Crombie and Whiting, 1998) thereby inhibiting mitochondrial respiration.

Of the 59 plants recorded in the ethnobotanical survey only 9 were used as topical antiseptics whereas 16 were used as either emetics, stomachics or to treat dysentery. The remaining plants were used in variety of roles including poisons, asthma type use and as analgesic/anti-inflammatory agents. It was claimed by the villagers that the water from the caldera lake in the centre of the peninsula was useful in treating tropical ulcers which are a common infective lesion in Papua New Guinea. It is conceivable that the extremes of pH found in such lakes could have produced an antibacterial effect thereby decreasing the emphasis on antiseptic plants that is found in other parts of Papua New Guinea. Many of the 59 plants assayed with the field kit gave rise to faint 1-2mm zones of inhibition but these were not considered sufficiently active to merit collection of samples.

3.3.1.2 Location 2. The foothills of the Whiteman Range

The second fieldwork site was centred on the village of Umbi in the foothills of the Whiteman Range at an altitude of approximately 500m. The local population who are an inland subgroup of the Kaulong linguistic group farm and hunt in forest up to approximately 1000m above sea level and so inhabit the ecotone between lowland forest and the lower margins of lower montane forest (Whitmore, 1984). The inland Kaulong are a small group numbering only a few hundred who practice a form of swidden agriculture made necessary by the poor soil quality which is a feature of the mountainous limestone karst terrain.

The Whiteman Range and its foothills are said to support an important tract of limestone flora and forest developed on sedimentary materials (Swartzendruber, 1993). The area is little explored botanically, but large tracts of Nothofagus forest are thought to occur on the higher plateaus (Swartzendruber, 1993).

46 plant species were recoded in the ethnobotanical field work and of these 19 were used to treat tropical ulcers, in each case either the sap from the relevant part of the plant or a mulch made from the plant was applied directly to the ulcer. In four cases plants were mixed with CaOH made by heating limestone from local deposits to form CaO and then adding water. Little mention was made by informants of non-plant derived medicines although the fat from a species of eel was reputedly used to treat *Tinea imbricata* skin infections. The most active plant species revealed with the field assay were a species of *Garcinia* (tentatively identified as *Garcinia dulcis* (Roxb. Kurz) known in the local Kaulong dialect as Kap and a bitter bark tree identified as *Lunasia amara* (Blanco) known locally as Avyái. Both trees were identified from infertile voucher specimens collected in forest within a 15km radius of the village of Umbi; the collection locations are shown below.

Lunasia amara (Blanco)	*Garcinia dulcis* (Roxb.) Kurz
05° 58.932S	06°01.231S
149°42.882E	149°47.493 (Position approximate)
Alt 538m	Alt 550m

Bark extracts of both trees provided clear zones of inhibition against *S. epidermidis* but not against *E. coli*. Informants had described the use of shavings from the bark of *Lunasia* amara and the yellow sap from the bark of *Garcinia dulcis* which were applied directly to tropical ulcers. Samples of bark from both trees were collected and shade dried.

3.3.1.3 Location 3. The Nakanai Mountains

The third location was centred on the mountainous region immediately east of the Nakanai plateau which provided extensive montane forest terrain above 1000m. The Nakanai Plateau is an uplifted limestone-capped formation reaching above 2000m at its highest point. It is the largest high altitude area in New Britain and is little surveyed and apparently biotically rich (Swartzendruber, 1993). The area is dominated by *Lithocarpus* and *Nothofagus* forest developed on the limestone substrate (Swartzendruber, 1993). The local population form part of the Mamusi ethno-linguistic group which extend from the south coast up in to the central mountains.

Respondents provided 19 species of plants and three species of insects that were used medicinally, of which 14 were used for the treatment of tropical ulcers. In the assay all but 6 preparations failed to give some sort of zone of inhibition with the field assay. One single preparation stood out amongst the other in terms of its activity which was a defensive secretion produced by a brightly coloured species of stick insect later identified as *Megacrania nigrosulfurea* (Redtenbacher).

When the insect is approached it fires either a fine spray or a stream of pungent smelling white fluid from glands located dorsally on the second segment of its body. 20µl of the secretion were assayed in wells giving zones of inhibition 22mm and 20mm in diameter with *S. epidermidis* and *E. coli* respectively. The local name in the Mamusi dialect for the insect is Kain tagho which translates literally as "eats *Pandanus* sp." (specimens of the insect that were caught in the field were generally found feeding on the leaves of a spiny bush tentatively identified as *Pandanus tectorius*). To gain sufficient quantities of the insect a specimen of *Pandanus tectorius* was uprooted and placed in an area of open cleared ground. Insects collected from the surrounding forest (see below for precise location) were then placed on the plant and were found not to stray from its shade.

Megacrania nigrosulfurea (Redtenbacher). 05° 40.004S
151° 05.551E
Alt 847m

Collecting the defensive secretion proved too difficult so when approximately 100 insects had been collected they were placed directly into ethanol for shipping to the UK.

3.4 The phagedenic ulcer

The tropical or phagedenic ulcer (Fig. 3.3) is a common infection amongst forest dwelling populations of Papua New Guinea and so featured occurred frequently in the ethnobotanical survey. The ulcer forms a distinct circular lesion of the skin causing necrosis of the dermal and epidermal layers that rapidly develops into a lesion a few centimetres in diameter within a few days (Adriaans and Drasar, 1987). The ulcer commonly affects lower parts of the leg which are particularly prone to infection after minor trauma such as a scratch or insect bite in the hot, humid tropical conditions; malnutrition is also thought to be a contributing factor (Braun-Falco, 1991).

The aetiology of the ulcer is not entirely clear although it is thought the ulceration may be preceded by a banal Streptococcal or Staphylococcal infection (Braun-Falco, 1991). The term tropical ulcer has been used to describe ulcerating infections caused by a variety of different organisms which in fact represent distinct diseases. These include the Buruli ulcer whose causative agent *Mycobacterium ulcerans* gives rise to sporadic outbreaks of infection as well as the rapidly developing frambesia ulcer associated with *Treponema pertenue* (Andrews, 1990).

Amongst the natives of New Guinea the terms used to describe tropical ulcers are no more discriminatory and as such it must be inferred that plants used to treat tropical ulcers are in fact used in the treatment of several distinct ulcerative infections.

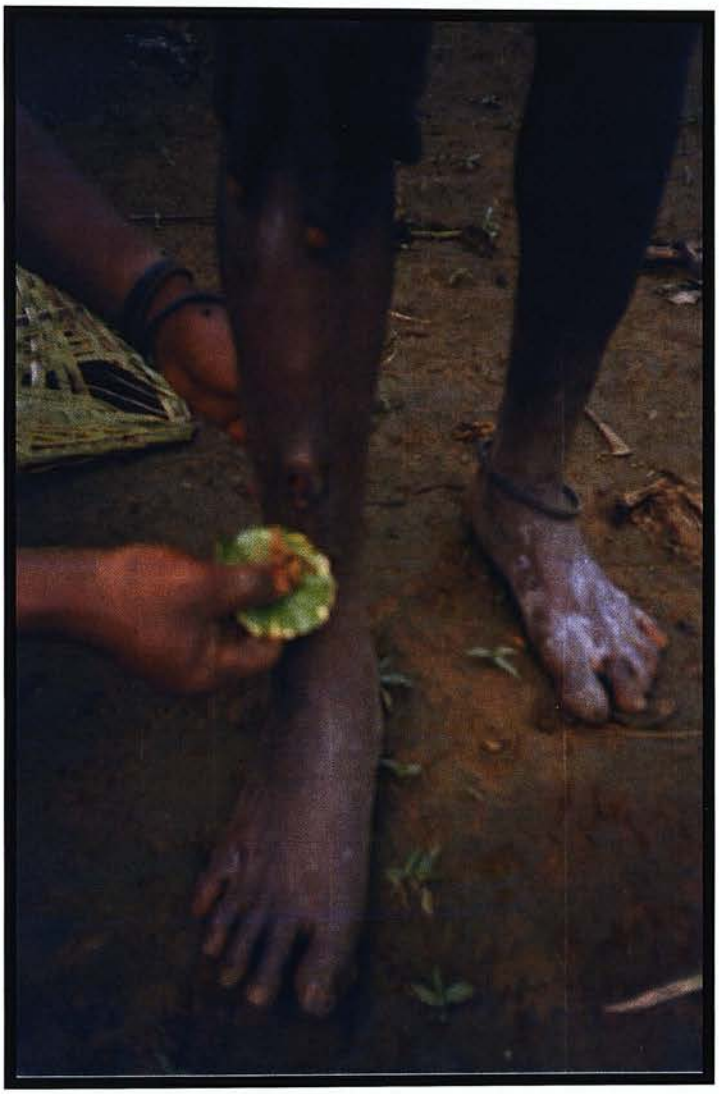


Figure 3.3. Application of *Lunasia amara* bark to a tropical ulcer. Fresh bark scrapings were placed in a leaf and applied directly to the eye of the ulcer.

The phagedenic tropical ulcer is thought to be caused by a polymicrobial infection with fusobacteria, aerobic microorganisms and spirochaetes each playing a role (Adriaans and Drasar, 1987). Adriaans et al (1987) isolated fusobacteria from samples taken from patients in Papua New Guinea, India, Zambia and Gambia revealed the presence of a species of *Fusobacterium* distinct from known members of the genus in terms of morphology, biochemistry and cell wall composition (Adriaans and Drasar, 1987). Spirochaete isolates have also been identified by electron microscopy as belonging to the genus treponemes (Adriaans and Drasar, 1987).

3.5 The active species

In total over 200 species of plants and 3 species of insects were screened using the field assay kit. Many of these gave small zones of inhibition (8-10mm including the well) but three in particular stood out as having clear antibacterial activity against one of the two test species; these were:

1. *Garcinia dulcis* (tentative identification)
2. *Megacrania nigrosulfurea* (Redtenbacher)
3. *Lunasia amara* (Blanco)

Garcinia dulcis and *Lunasia amara* were identified by Robert Kiapranis of the Forest Research Institute Papua New Guinea from infertile voucher specimens whilst the phasmid insect *Megacrania nigrosulfurea* was identified by Dr Oliver Zompro of the Max Planck Institute for Limnology, Germany from specimens collected into 100% methanol and colour photographs.

3.5.1 Garcinia dulcis

Garcinia dulcis (Roxb.) Kurz is a medium sized tree with smooth evergreen leaves which produces an edible fruit a few inches in diameter. The *Garcinia* genus falls within the Guttiferae and consists of 180 species of mostly evergreen trees distributed throughout tropical Asia, Africa and Polynesia (Ansari et al., 1976).

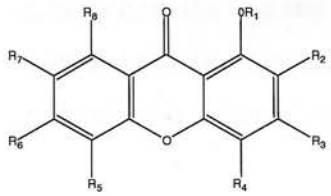
Figure 3.4. *Garcinia dulcis*. Taken from the internet site http://www.tradewindsfruit.com/order_fruit_f_o.htm

The genus has attracted considerable attention in recent years as a source of novel flavanoids, benzophenones and xanthones (Harrison, 1994). The bark is known in Thailand for its antiseptic properties and in Indonesia its leaves are used in the treatment of various disorders including lymphatitis, parotitis and struma (Likhitwitayawuid et al., 1998). Xanthones, a type of flavanoid are widely distributed in the genus and have been associated with a variety of pharmacological activities including antibacterial, antifungal and antimalarial activity (Kosela et al., 2000). Rubraxanthone isolated from *Garcinia dioica* has what is thought to be the lowest MIC for MRSA strains (0.31-1.25μg/ml) recorded for a plant derived compound (Iinuma et al., 1996).

A number of compounds have been isolated from *G. dulcis* (Fig. 3.5); Ansari et al (1976) isolated a series of biflavanoids and a flavanone-chromone from the leaves (Ansari et al., 1976) whilst extraction of the branches yielded a novel xanthone, the triterpenoid friedelin and three flavanoids 3´-(3-methylbut-2-enyl)naringenin, I3,II8-biapigenin and podocarpusflavoneA (Harrison, 1994). Five further xanthones were isolated from the bark all of which displayed inhibitory activity towards the malarial parasite *Plasmodium falciparum*, with IC_{50} values of between 0.96 and 3.88μg/ml (Likhitwitayawuid et al., 1998). A further four dulxanthones were isolated from the leaves by Kosela et al (Kosela, 1999), (Kosela et al., 2000).

A quantity of the bark was collected for laboratory analysis and once in the lab it was apparent the active principles were extremely hydrophobic since they readily extracted into hexane but not at all into water. A hexane extract of the bark re-suspended in methanol gave MICs of 1μg/ml against both sensitive and drug resistant (MRSA) strains of *S. aureus* (NCTC 6751 and NCTC 12493) using the standard agar dilution method for sensitivity testing stipulated by the British Society for Antimicrobial Chemotherapy (Andrews, 2001). A variety of extraction and separation schemes were applied to the bark including normal phase HPLC using hexane and ethylacetate as eluents but none of these afforded fractions of sufficient purity to allow structural characterisation of active principles. Thus despite the promising antibacterial activity of the bark extracts the

inherent limitations of normal phase HPLC meant that isolation of pure compounds would prove unlikely and further work with the plant was not carried out.



	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
1	H	H	H	H	H	H	H	H
2	H		H	OH	OH	H	H	H
3	Me	OH	H		OH	OH	H	H
4	H	OH	H		OH	OH	H	H
5	H	H	OH	H	OH	OH		

Figure 3.5 Xanthones isolated from the bark of *G. dulcis* by Harrison et al (Harrison, 1994). Complete side chains including the –R stem are shown for clarity.

3.5.2 Megacrania nigrosulfurea

Megacrania nigrosulfurea (Redtenbacher) belongs to the order Phasmatodea which includes both the leaf and stick insects (Redtenbacher, 1908). There are at least 3,000 described species of phasmid the majority of which occur in the tropics; they are predominantly arboreal and nocturnal (Whiting et al., 2003).

Figure 3.6. *Megacrania nigrosulfurea* (Redtenbacher) adult and nymph on a *Pandanus* sp. leaf.

The name phasmid is derived from their ability to camouflage themselves with their surroundings and as such they are characterised by the use of morphological and behavioural crypsis to mimic sticks and leaves (Whiting et al., 2003). In the case of *M. nigrosulfurea* however the black and orange and black and yellow stripes seem to indicate an aposematic colouring scheme. A second morphological characteristic of the Phasmatodea is the presence of prothoracic repellent glands (Whiting et al., 2003) which can be used in one of two ways, either to spray a narrow jet of fluid at the predator itself or to spray a fine mist of the defensive secretion so as to cover the body of the insect and make it unpalatable to the predator (Bouchard, 1997).

M. nigrosulfurea is only known from the type material collected by Redtenbacher in 1908 and therefore until rediscovered during this fieldwork project had not been recorded in almost 100 years (personal communication Dr Oliver Zompro). As such there is no information on compounds derived from the species but analysis of the defensive secretions of other insects in the order have been carried out. Chow et al (1986) isolated five odour compounds from the secretions of the related species *Megacrania alpheus* and identified the major component as the monoterpene alkaloid actinidine (Fig. 3.7) which has also been isolated from the rove beetle, dolichoderine ants and the Argentine ant (Chow, 1986). They also determined that insects deprived of the secretion stood a greatly reduced chance of surviving to maturity indicating an adaptive role for the secretion.

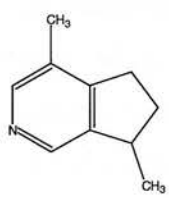


Figure 3.7 Actinidine isolated from the defensive secretions of *Megacrania alpheus.*

More recently, eight constituents were isolated two of which were revealed to be stereoisomers of 1-acetyl-3-methylcyclopentane and the remaining six were tentatively assigned as derivatives of actinidine by comparison with authentic samples on GC-MS (Ho, 1993). Intriguingly, actinidine was also found in the leaves of the insect's dietary plant *Pandanus tectorius* suggesting the phasmid not only concentrates the alkaloid to form its secretion but chemically modifies it as well (Ho, 1993). Other constituents isolated from insects within the order include the monoterpenes iridodial and nepetalactone along with benzaldehyde, benzothiazole, limonene, diethyl ether and acetic acid (Bouchard, 1997).

A number of the insects were collected for analysis rather than the defensive secretion itself which proved too problematic to collect and these were later tested along with the methanol the insects were collected into but the antibacterial activity could no longer be detected.

2.5.3 Lunasia amara

The genus *Lunasia* (Blanco) is almost exclusively Malesian; specimens described by Hartely in his revision of the genus have been found throughout the Philippines, Borneo and Java through to Papua New Guinea as well as the Cape York Peninsula of Australia (Hartley, 1967). Members of the genus are typically dioecious, evergreen erect shrubs or small trees and are distinguished by their trimerous flowers arranged in small head like clusters which do not occur in other genera of the Rutaceae. Although *Lunasia* is extremely variable in certain vegetative features these do not correlate to specific ecological habitats and as such, variant sub-types to do not represent distinct species (Hartley, 1967). Thus various members of the genus such as *L. costulata* and *L. quercifolia* are in fact all the same species *Lunasia amara*. Hartly recognises two varieties; *L. amara*. Var. *amara* occurs throughout Malesia whereas var. *babuyanica* is restricted to the Philippines. Hartley states that the genus is confined entirely to

lowlands (up to 900m) and grows in habitats ranging from well drained rain forests to secondary re-growth and dry thickets. In contrast to this, samples of *Lunasia* collected for this project were all observed to be found growing next to or partly submerged in fast flowing water (Fig. 3.8).

Figure 3.8 *Lunasia amara* (Rutaceae) is a small tree up to approximately 5m in height with variable vegetative features. The wood is very hard and the bark extremely bitter tasting.

Lunasia spp. are reported to be used in native medicines of the Philippines and Indonesia for the treatment of snake bite, skin diseases, swollen limbs, inflamed eyes as well as digestive disorders for which it is taken internally but only in small quantities otherwise side effects such as cramps and vomiting are said to occur (Hartley, 1967). There are several reports of *Lunasia* barks being used as arrow poisons (Hartley, 1967) but later investigations by Brill and Wells (1917) were unable find any evidence to support this (Wells, 1917). The story is thought to have emerged as a result of shipments of

Lophopetalum toxicum mis-labelled as *Lunasia amara* being sent to European laboratories at the beginning of the 20th century (Wirth, 1931). This taxanomic error seems to have been compounded by Blanco's original description of the species in which he states "The grain is very fine and close, and the wood is so hard that the Negritos use it instead of iron for the points of their arrows."

Father Juan J. Delgado a catholic missionary writing on the indigenous uses of Philippino plants in 1892 describes the use of certain *Lunasia* spp. referred to locally as Paetan, in treating infected wounds. He states, "it is admirable for infected wounds, for it will clean and cure them in a short time" (Delgado, 1892). He also describes how "merely chewing a small bit of its bark, I was hardly able to that day to remove the bitterness from the tongue, experiencing some sort of convulsions for three days" (Delgado, 1892). Delgado translates Paetan to mean "bitterness itself", perhaps a reflection on the high alkaloid content of the bark.

Various studies have been carried out into the phytochemical constituents of different *Lunasia spp.* and a number of compounds have been isolated and their structures determined. Of all the plant families the Rutaceae is the most prolific in terms of the structural variety of alkaloids it produces which include acridines, furoquinolines, quinolines, quinazolines, indoquinazolines, canthinones, imadazoles, benzoisoquinolines and aromatic amines and amides (Price, 1963). From the bark and leaves of *L. amara* 13 quinoline type alkaloids have been isolated (Fig. 3.9) starting as far back as 1899. The bark is particularly rich in alkaloids with an alkaloid content reported to range from 4 to 4.5 % for Australian samples (Johnstone, 1958).

2.5.3.1 Compounds isolated from the bark of *Lunasia amara*

The stem bark contains the alkaloid lunasine as the principal alkaloid (Collins, 1990) together with 12 other structurally related quinoline type alkaloids. Boorsma isolated lunasine, lunacrine and lunacridine (Henry, 1939) whilst Steldt and Chen (1943) isolated lunacrine, lunacridine, lunamarine, lunamaridine by sequential extraction with a range of solvents followed by crystallisation but lunasine was not isolated which the author states may be due to its decomposition to lunacridine upon reaction with NaOH in the extraction solvents (Steldt, 1943). Johnstone et al (1958) isolated lunacrine, lunine, and a new alkaloid eduleine from the Australian variety *L. quercifolia* now recognised as the same species as *L. amara* by filtering a benzene extract though aluminia followed by numerous rounds of acid/base extraction and crystallisation (Johnstone, 1958). In their samples lunacrine was the major alkaloidal component. Price (1959) isolated lunasine by reaction of the water soluble bases with picric acid followed by several rounds of crystallisation and using UV and IR spectroscopy was able to determine the structures of lunasine and lunacridine (Price, 1959). Ruegger and Stauffacher (1963) isolated hydroxylunidine, hydroxylunacridine, lunacridine, lunacrine as well as two new alkaloids lunidine and lunidonine by alumnia chromatography eluting with increasingly polar solutions of benzene and methanol (Ruegger, 1963).

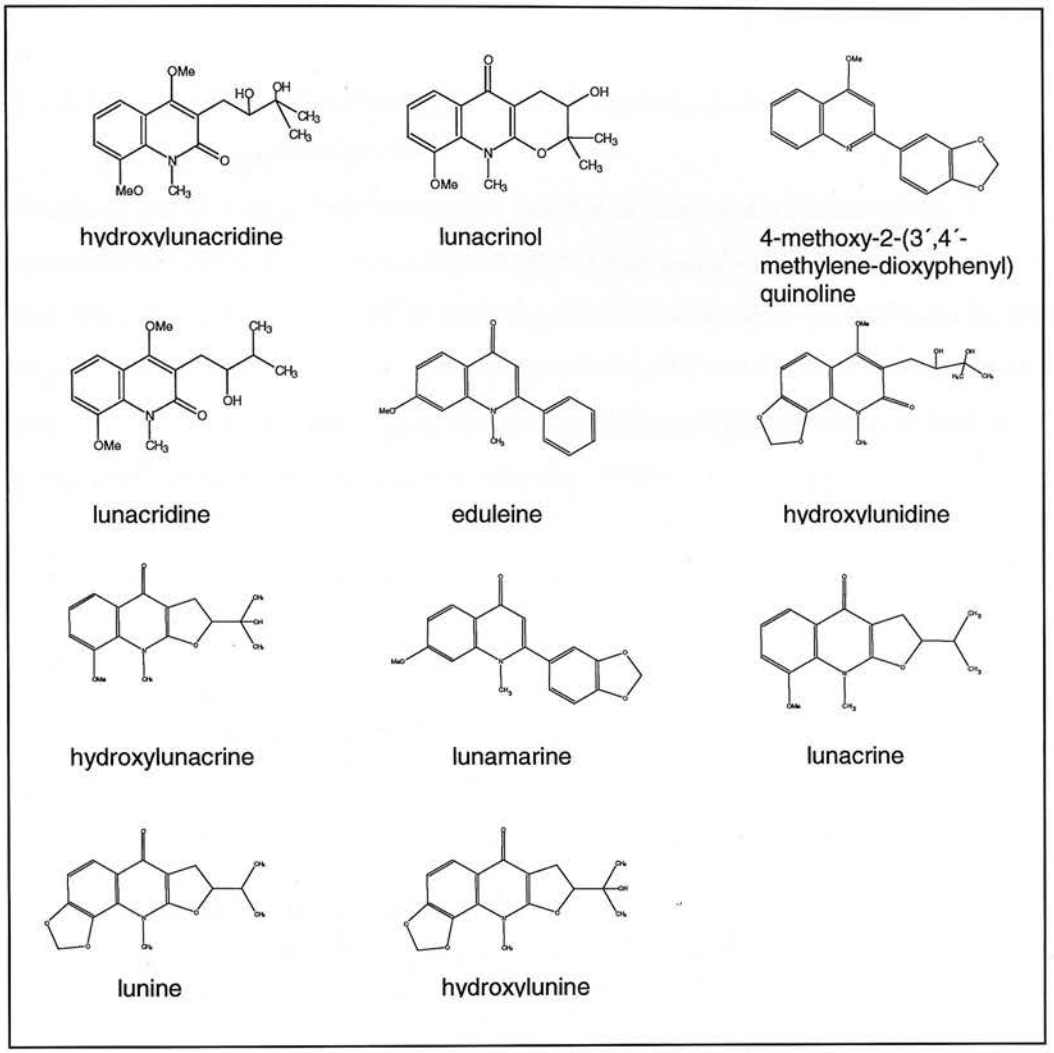


Figure 3.9 Compounds isolated from the bark of *Lunasia amara*

2.5.3.2 Compounds isolated from the leaves of *Lunasia amara*

Goodwin and Horning (1957) isolated a number of alkaloids from the leaves. 7 compounds were eluted from aluminia but only one 4 methoxy 2 phenyl quinoline was described (Goodwin, 1957). More recently, steam distillation of the leaves by Brophy et al produced an oil which when analysed by gas GC-MS was revealed to be almost entirely composed of sesquiterpenoids, the principle components being γ-elemene, germacrine-D and bicyclogermacrine (Brophy, 1997).

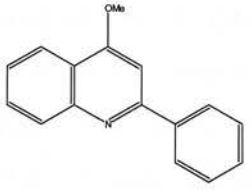


Figure 3.10 4-methoxy-2-phenyl-quinoline

2.5.3.3 Biological activities of *L. amara* alkaloids

In a 1955 survey of 150 flowering plants collected in Queensland, Australia and New Guinea and tested for antibacterial activity, *L. amara* bark appears as one of the two most active species producing 3-5mm zone of inhibition for *Salmonella typhi* S76 and > 7mm for *S. aureus* B313 and *Mycobacterium phlei* CSL (Atkinson, 1956). Indeed, of the 18 species of Rutaceae tested, only one failed to show any antibacterial activity. The methods used however included applying dried plant material in unspecified amounts directly to agar plates so cannot be considered in any way quantitative.

The alkaloids lunacrine and lunasine were studied by Wirth in 1931 for their pharmacological activity. Both alkaloids show a similar profile in terms of their action on various muscle tissues but lunasine is the more potent of the two (Wirth, 1931). The two alkaloids were found to have a stimulatory effect on voluntary and smooth muscle as well as stimulating the contraction of blood vessel walls and decreasing cardiac output but no anaesthetic action on sensory or motor nerves and or the sympathetic ganglion was found (Wirth, 1931). Wirth also indicates the two alkaloids have an inhibitory effect on certain protozoa but from the 1931 text it is not clear what species or at what concentration the stated antimicrobial effects occur.

Steldt and Chen (1943) investigated the cardiac effects of lunacrine, lunacridine, lunamarine and lunamaridine and found none of the four alkaloids to cause systolic standstill when injected into the ventral lymph sac of a frogs heart thereby disproving the notion of Lunasia alkaloids being cardiac poisons with a digitalis like actions as had been indicated by some authors (Steldt, 1943). In etherised cats intravenous injections of lunacrine hydrochloride 15mg or lunacridine 10mg caused a moderate fall in blood pressure with recovery occurring within 2 minutes and a reduction in the amplitude of respiration. Injection of lunamarine resulted in an initial drop in carotid pressure followed by a secondary increase (Steldt, 1943). Lunamarine was also found to stimulate isolated rabbits intestine and uterus at a concentration of 1:200 000. No significant changes in blood sugar concentration were found to occur when three of the alkaloids lunacrine 50mg/kg, lunacridine 5mg/kg lunamarine 0.4mg/kg were injected into rabbits (Steldt, 1943).

Toxicity experiments were also carried out to determine the median lethal dose in albino mice of each compound. The median lethal dose of lunacrine hydrochloride by IV injection was 78.7mg/kg whilst the median lethal dose of lunacridine by mouth was 1097mg/kg. Doses as high as 1000mg/kg lunamarine were not fatal (Steldt, 1943).

When a fraction of *L. amara* water soluble bases was tested on mice, doses of 50 to 100 mg/kg produced ledge unsteadiness and ataxia whilst 500mg/kg resulted in death in 1 to 5 hours (Collins, 1990). In anaesthetised cats, doses of 20 or 40mg/kg induced cardiovascular depression for 10-20 minutes whilst in isolated rabbit ileum 0.001-0.01mg/ml antagonised furmethide-induced spasm. The fraction displayed weak antimicrobial activity against *Micrococcus pyrogenes*, *Diplococcus pneumoniae*, *Klebsiela pneumoniae* and *Trichophyton mentagrophytes* at 120µg/ml but was inactive against Newcastle disease virus and adenovirus type 4 (Collins, 1990). Furoquinolines similar to those found in *Lunasia* Spp. have been associated with phototoxicity towards bacteria, viruses, yeasts and CHO cells. Their mechanism of action is thought to involve the formation of photoadducts with DNA (Paulini et al., 1989).

Several kilograms of *Lunasia amara* bark were collected for laboratory testing and due to the general paucity of information concerning the antimicrobial activity of *Lunasia* alkaloids it was decided to investigate further the active constituents of the bark.

Chapter 4

PURIFICATION AND IDENTIFICATION OF ACTIVE PRINCIPLES

4.1 Purification by open column chromatography

Open column or low pressure chromatography are terms used to describe all forms of chromatography in which the movement of the mobile phase though the stationary phase is gravity fed (Robards, 1994). It is the oldest from of chromatography and one of the cheapest to perform since it does not require the highly engineered parts found in closed systems such as HPLC and GC. Since different analytes in the sample mixture have varying affinities for the mobile and stationary phases they separate out as they pass through the column and are eluted at different time intervals.

The role for medium pressure chromatography in natural product separations should be considered an initial cleanup step before more powerful separation methods such as HPLC are applied. By reducing the amount of material loaded onto HPLC columns, more efficient separations can be achieved. Samples chromatographed through open column media have the added advantage that fine particles as well as high molecular weight compounds such as tannins and proteins have been removed. Where the composition of the sample mixture is not known or in this case the compound of interest is unknown, it is impossible to accurately predict which conditions will favour a good separation so this must be determined through trial and error followed by successive rounds of optimisation.

Various chromatographies were trailed in a search for methods that could be combined to form a protocol for the activity-guided purification of the active principle(s). In each case, fractions were collected and assayed to check if the activity had been released from the column and elution profile graphs were produced to determine the quality of the separation. For convenience only a fraction of all the separations carried out are depicted in this section.

The separation techniques employed generated a large number of pure or partly pure fractions and so a simple easily reproducible assay was therefore needed to detect the

activity in each fraction. The well method in which up to 100µl of each fraction is injected into pre-cut wells 6mm in diameter on plates inoculated with a bacterial culture (*S. aureus* NCTC 6571) was found to work well with un-concentrated fractions whilst fractions with higher activity were assayed using the disk diffusion method.

4.2 Optimising the extraction protocol

Before starting the purification of the active principle(s) it was necessary to optimise the extraction of the bark to form a crude extract that contained the all the antibacterial activity of the bark in the smallest possible volume of solvent. Solvents of increasing hydrophobicity were tried revealing ethanol and water as possible candidates (Fig. 4.1). These were then used at a range of proportions of solvent to bark (ground to a fine powder) and after each extraction the extracted bark was re-extracted and assayed to determine how much residual activity remained (Fig. 4.2). The minimal volume of solvent that left no residual activity in the bark was then adopted for all future experiments.

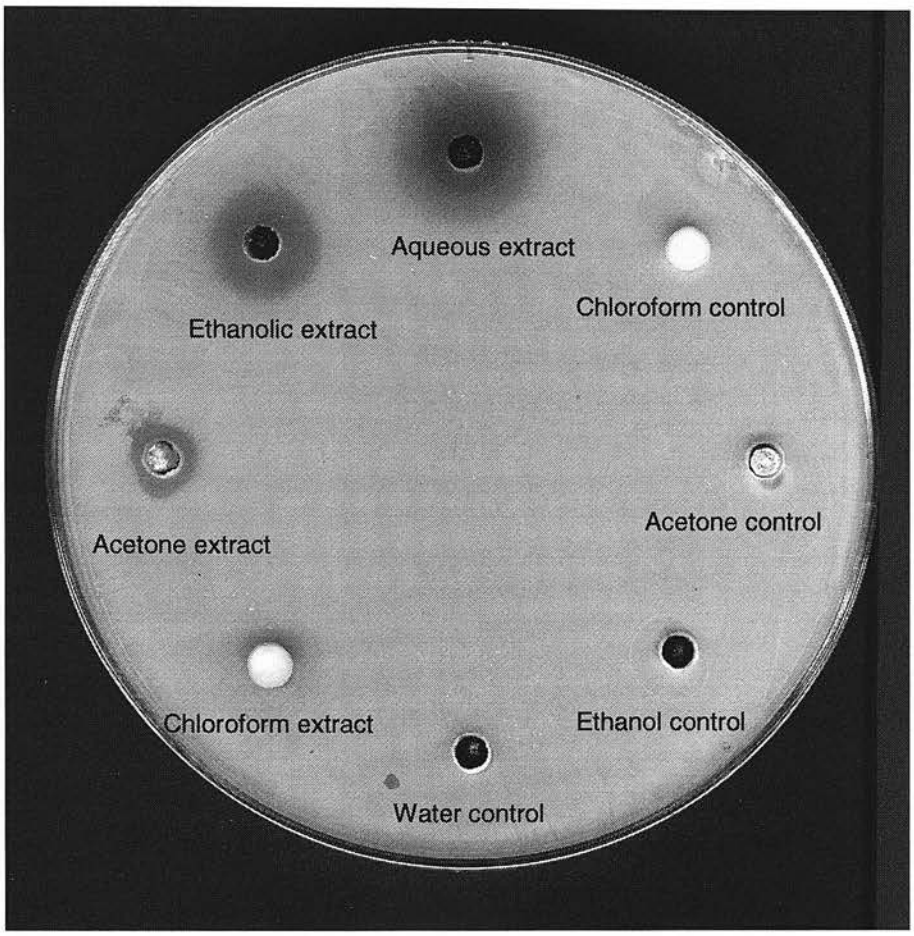


Figure 4.1 Aqueous, ethanolic, acetone and chloroform extracts made by extracting 100mg of powdered *L. amara* bark with 1 ml of water, ethanol, acetone and chloroform were applied to a plate inoculated with *S. aureus* NCTC 6571. 50µl of each extract and solvent alone as controls were injected into wells and the plate incubated overnight.

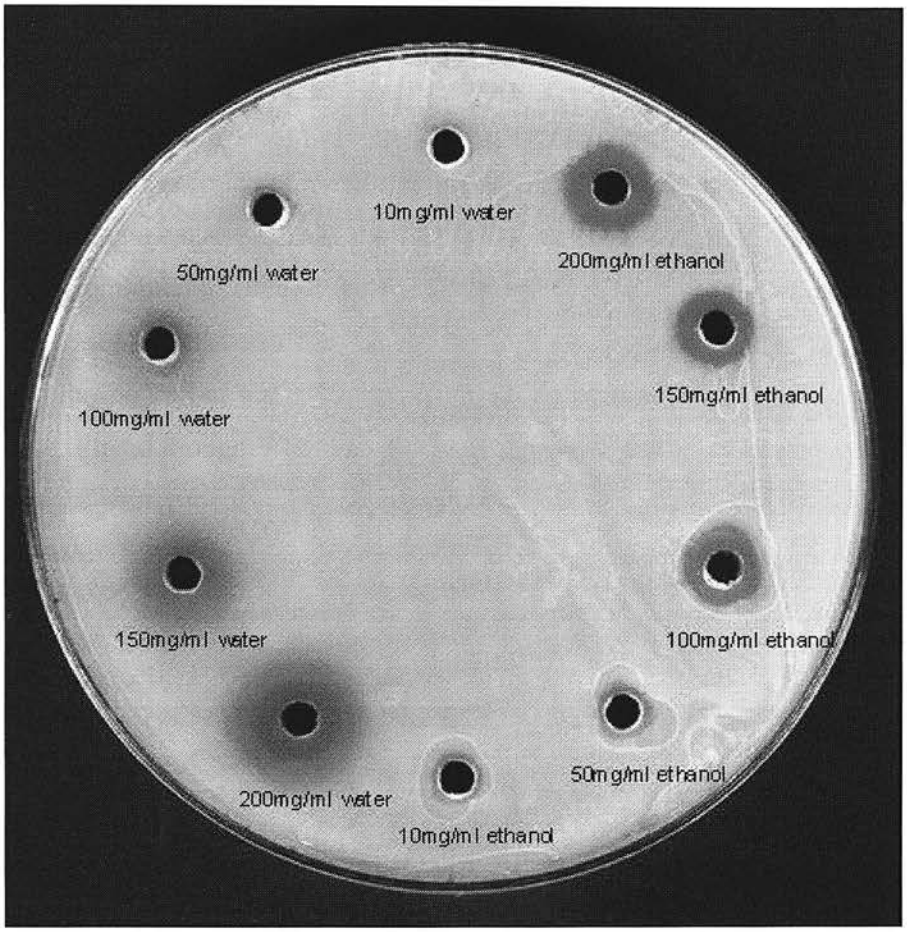


Figure 4.2 Determining the optimal extraction conditions. Ethanolic and aqueous extracts were made with varying ratios of bark powder to solvent. After vortexing thoroughly the extract was centrifuged and the extract removed. The extracted bark powder was then re-extracted with 1ml solvent and 50µl assayed in wells to detect residual activity left behind from the first extraction.

The plates suggest water and ethanol as suitable extraction solvents and in each case 50mg bark powder per 1ml solvent is the highest extraction ratio that can be used without leaving behind residual activity in the bark.

4.3 Trialling different open column techniques

A variety of separation techniques were applied to the *L. amara* bark crude extract including adsorption chromatography on silica, gel filtration with Sephadex LH-20, and ion exchange using DEAE and CM52 resins. The most effective chromatographies were then combined to attempt the complete purification of the active principle(s). In the following experiments dried *L. amara* bark was ground in a mortar and pestle in the proportion 1ml of water/50mg dried bark. This produced a dark coloured liquid which was filtered though Whatman No 1 filter paper to remove fine particles. Elution profile graphs were produced by recording UV absorption (optical density) for each fraction at 280nm.

4.3.1 Silica adsorption chromatography

Classical silica column chromatography has been used with some success in the isolation of plant alkaloids so it was decided to begin with this approach. The separation mode is by adsorption chromatography in which solute molecules compete for active sites on the stationary phase which is coated in a layer of mobile phase molecules (Snyder, 1968). Solute retention occurs by either association with or displacement of the adsorbed mobile phase molecules. As can be seen from the elution profile graph below however, elution with ethanol/water 4:1 afforded very minimal separation of the bark extract.

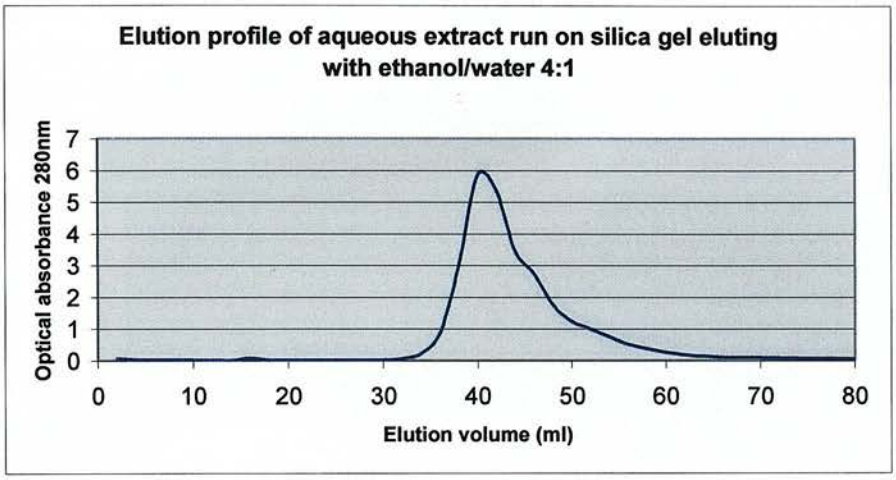


Figure 4.3 Silica Gel was swelled in ethanol/water 4:1 and then poured into a plastic column and allowed to bed out (bed size 2.4cm x 11.3cm) before running with more solvent to wash away impurities. 4ml of the aqueous bark extract were then loaded into the column and eluted with 80ml ethanol/water 4:1 collecting fractions automatically every 2ml and recording UV absorbance (optical density) at 280nm.

Each fraction was concentrated to approximately 300µl volume and 80µl of each assayed in wells but none of the fractions contained any activity.

The experiment was repeated eluting with methanol/water 4:1 to see if this would improve the separation but after assaying each fraction in wells there was still no detectible activity.

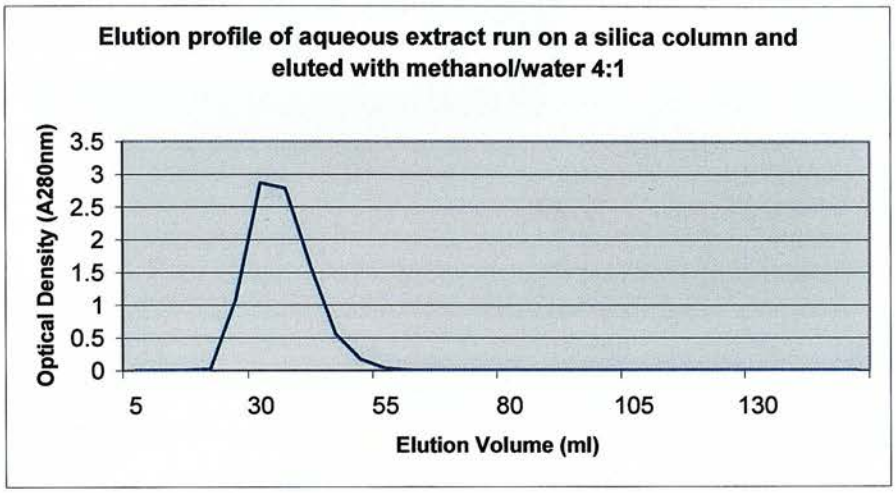


Figure 4.4 Separation of crude extract on silica. Bed size of column was 2.7cm x 3.8cm and eighty 5ml fractions were collected.

Next, to try and reduce the strong adsorption seen with standard silica gel, a column was set up using Florisil (activated Magnesium Silicate) and ran as before collecting fifty 3ml fractions.100µl of each fraction were assayed in wells but as before none of the fractions contained any activity.

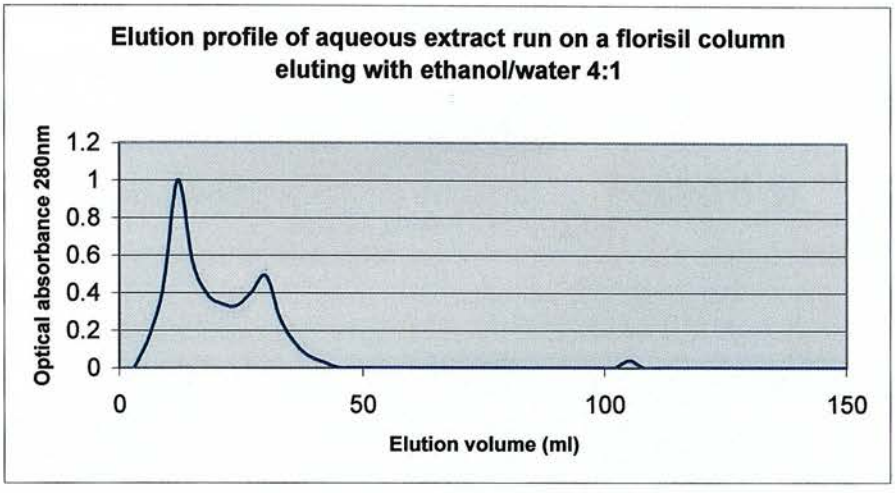


Figure 4.5 Florisil powder was swelled in methanol/water 4:1 and then poured into a plastic column and allowed to bed out (bed volume 1.6cm x 7.5cm) before running with more solvent to wash away impurities. 2ml of the aqueous bark extract were then loaded into the column and eluted with 150ml methanol/water 4:1 collecting fractions automatically every 3ml and recording UV absorbance at 280nm.

4.3.2 Sephadex LH-20 gel filtration chromatography

Sephadex LH-20 is a dextran-based gel that separates compounds according to size (gel filtration) and is ideally suited to the separation of small molecule natural products such as steroids, terpenoids, lipids and low molecular weight peptides (Pharmacia, 1987). LH-20 is manufactured by hydroxyl propylation of cross linked dextrans which are formed into beads 23-163 microns in diameter when swelled in methanol (Pharmacia, 1987). The dextran beads contain numerous pores which small molecules may permeate into with varying ease depending on their size. Large molecules do not enter the pore at all and are eluted at the volume of solvent equivalent to the interstitial column volume whereas intermediate sized molecules are able to permeate into the pores and are eluted at varying intervals depending on their size. Thus LH-20 acts as a kind of reverse sieve in which larger compounds may elute before smaller ones.

A Sephadex LH-20 column was tried next to see if it would give some degree of separation without the strong adsorption seen with silica.

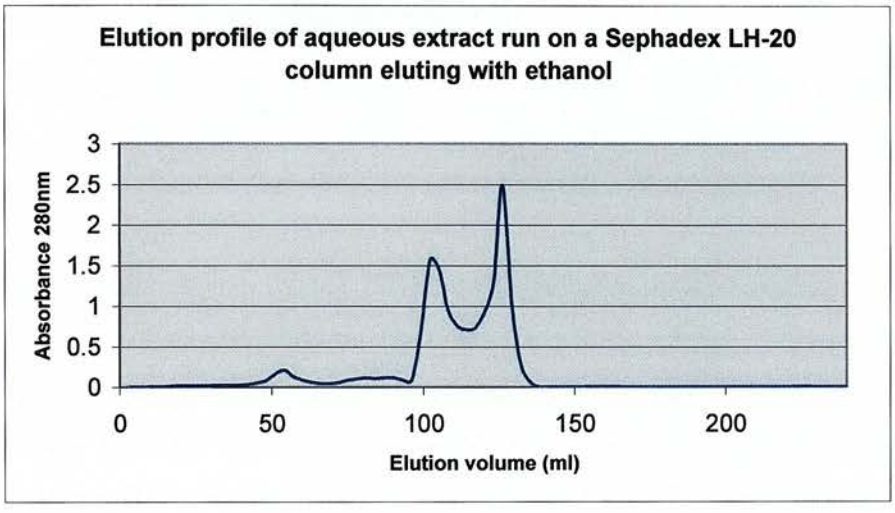


Figure 4.6 Sephadex LH-20 was swelled in ethanol and poured into a plastic column to form a bed size of 2.5cm x 40cm. 2ml ethanolic bark extract pre-filtered through a 0.20µm disposable syringe filter were loaded into the column which was then eluted with ethanol collecting eighty 3ml fractions. UV absorbance at 280nm was recorded for each fraction.

1ml of each fraction were evaporated to dryness and taken up in 50µl water which was then assayed in wells. Two fractions corresponding to 105-108ml elution volumes gave zones of inhibition indicating the active principles were eluted from the column in the first of the two large peaks.

Elution volume	Zones of inhibition
0-104	0mm
105	18mm
108	18mm
109-240	0mm

Table 4.1 Zones of inhibition from LH-20 gel filtration fractions.

Thus activity was released from the column which afforded some separation of the bark extract suggesting LH-20 gel filtration as a potential step in the purification of the active principles.

4.3.3 Diethylaminoethyl-cellulose anion exchange chromatography

Diethylaminoethyl-cellulose (DEAE-cellulose) is an anion exchange media useful in the separation of sample analytes containing negatively charged compounds which may bind to its positively charged amide group. A Dowex anion exchange column (bed volume 19cm x 1.7cm) was set up and equilibrated in 10mM pH 7.0 HCl Tris buffer. The column was loaded with 2ml of the aqueous bark extract and eluted with a flow-through of 50ml 10mM Tris buffer collecting fifty1ml fractions. The column was then eluted with a salt gradient to wash off bound material. A gradient maker was set up with 25ml 1M NaCl 10mM pH 7.0 Tris in the high salt chamber and 25ml 10mM pH 7.0 Tris in the low salt chamber. The column was eluted with the resulting salt gradient until eighty 1ml fractions had been collected. A dark band was still visible at the top of the column so a further elution was carried out with 25ml 3.0M $MgCl_2$ 10mM pH 7.0 Tris. Each fraction was concentrated to 300µl and 80µl from each, assayed in wells.

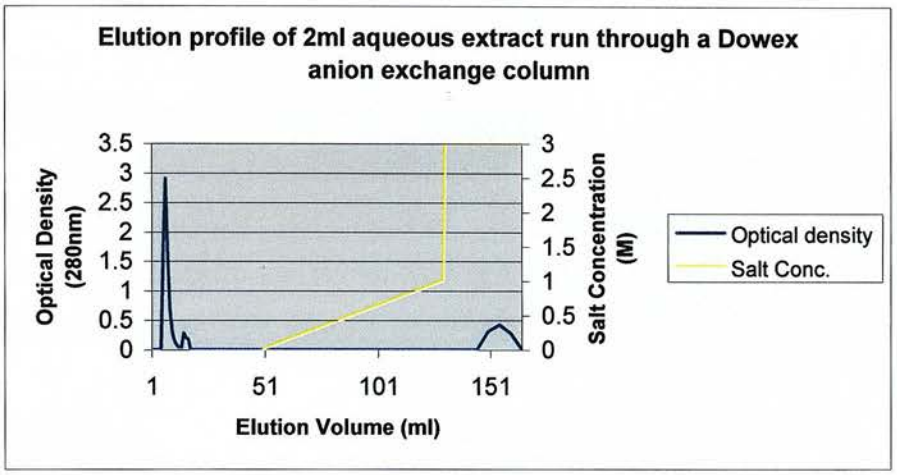


Figure 4.7 Anion exchange chromatography of aqueous bark extract eluted with a pH 7.0 flow through followed by a 0 to 1M KCl salt gradient and a 3.0M $MgCl_2$ salt wash.

Elution volume	Zones of inhibition
0-5	0
6	16
7	19
8	16
9	11
10-155	0

Table 4.2 Zones of inhibition from dowex anion exchange fractions.

The assay reveals the active principle(s) to be eluted in the first peak of the flow through; therefore they do not bind to the column. There is however some degree of separation as three distinct peaks are present.

4.3.4 CM52 cation exchange chromatography

Since anion exchange chromatography failed to bind the active principles the ion exchange procedure was repeated using a carboxy methyl cellulose based resin (CM52) which binds positively charged species.

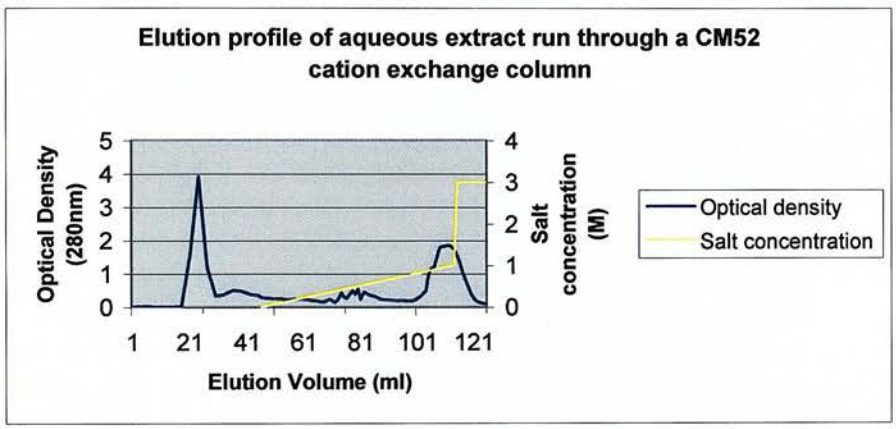


Figure 4.8 A CM52 column (bed size 2.7 x 7cm) was set up and equilibrated with 200ml 1M pH 7.0 Tris followed by 500ml 10mM pH 7.0 Tris. 2ml of the aqueous bark extract was loaded into the column and a flow through carried out in which the column was eluted with 10mM Tris collecting fifteen 3ml fractions. A salt gradient was then set up in which the low salt chamber contained 10 mM Tris and the high salt chamber 1.0 M NaCl 10mM Tris. The salt gradient was run for seventy 1ml fractions whereupon the column was eluted with 3.0 M $MgCl_2$ to elute any remaining bound material.

Elution volume	Zones of Inhibition
Flow through 1-45	0
Salt gradient 46-115	0
High salt elution 116-125	0

Table 4.3 Zones of inhibition from CM52 ion exchange fractions.

The results show the active principle(s) were not released from the column even after elution with 3.0M $MgCl_2$ suggesting a strong interaction with the column media and the presence of a positive charge. The experiment was therefore repeated with increasing salt concentrations during equilibration to try and decrease the binding capacity of the column. The column bed size was kept the same throughout.

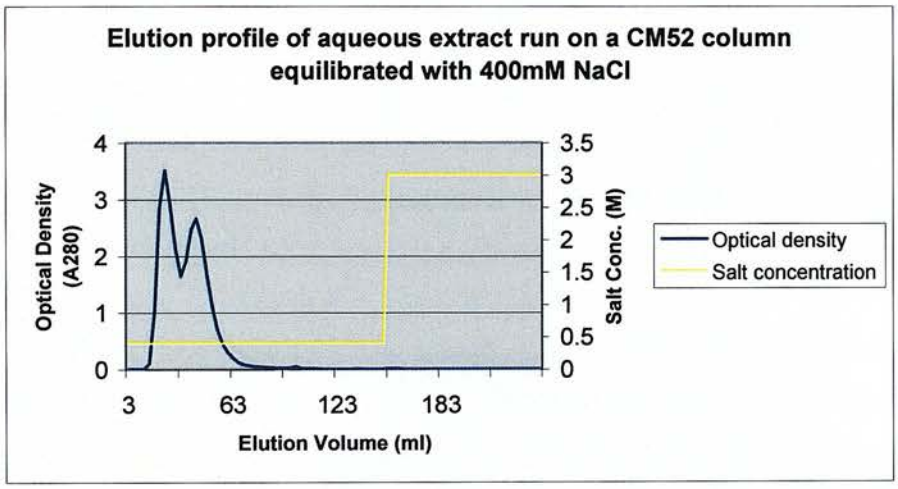


Figure 4.9 Elution profile of 2ml aqueous extract run on a CM52 with a 200mM NaCl equilibration followed by 3.0M $MgCl_2$ elution.

No activity was recovered so the experiment was repeated equilibrating the column with 2.0M NaCl 10mM pH 7.0 Tris.

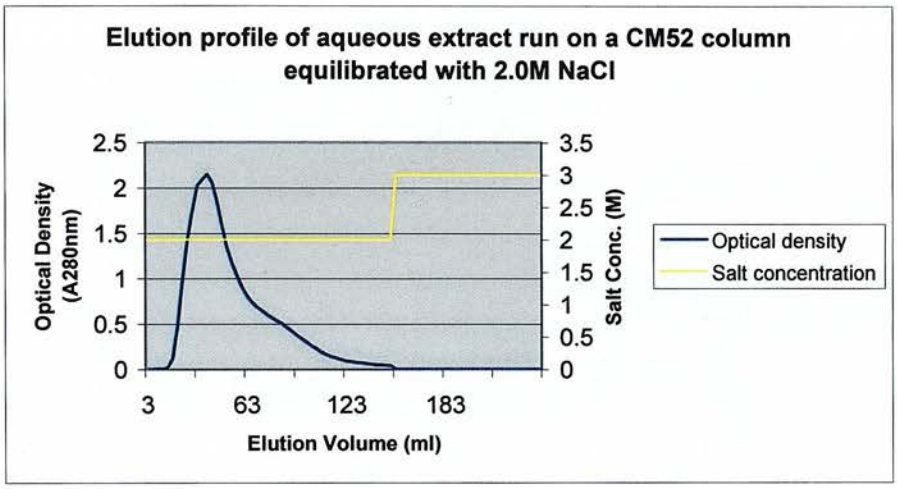


Figure 4.10 Elution profile of 2ml aqueous extract run on CM52 with a 2.0M NaCl equilibration followed by 3.0M $MgCl_2$ elution.

As with previous experiments no activity was released from the column demonstrating that even high salt equilibrations are unable to remove enough binding capacity from the column to allow elution of the active principle(s). An alternative approach was needed so the experiment was repeated at a range of pH levels to see if this would weaken the interaction through ion-suppression.

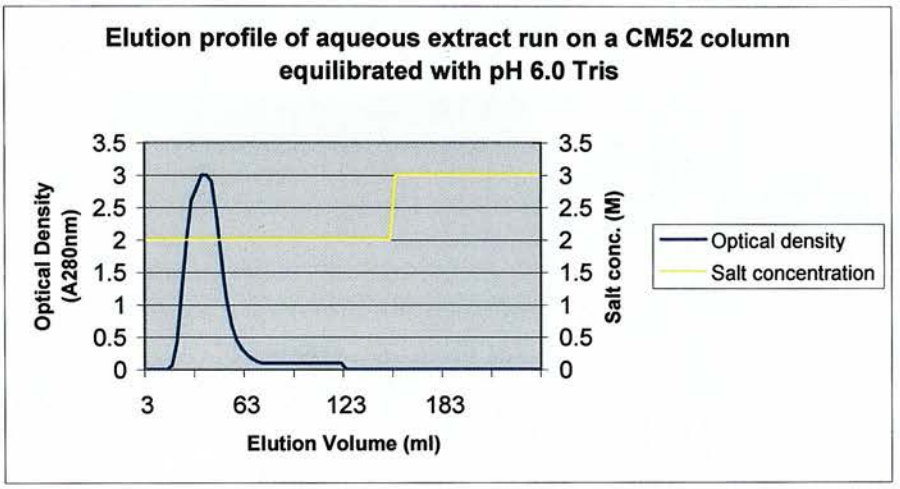


Figure 4.11 A CM52 column was equilibrated with 1.0M pH 6.0 Tris followed by 10mM pH6.0 Tris. Fifty 3ml fractions were collected eluting with 10mM pH 6.0 Tris 2.0M NaCl and a further 30 fractions were collected eluting with 3.0M $MgCl_2$ 10mM pH 6.0 Tris.

The lowered pH failed to release the active principles from the column so the experiment was repeated at pH 2.0 using citric acid HCl as the elution buffer. The buffer was made by dissolving sodium citrate powder in water and adjusting the pH with HCl. After equilibrating the column with pH 2 2.0M NaCl it was eluted with 150ml of the same buffer collecting fifty 3ml fractions.

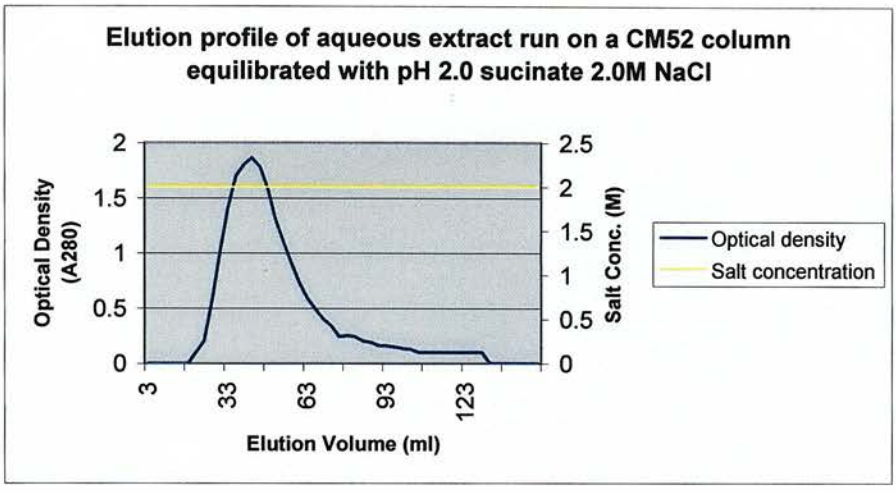


Figure 4.12 Elution profile of 2ml aqueous extract run on CM52 with a 2.0M NaCl pH 6.0 equilibration followed by 3.0M $MgCl_2$ pH 6.0 elution.

Further lowering the pH failed to release the active principles so a higher pH elution was carried out to see if this would suppress some of the positive charge associated with the active principles.

The experiment was repeated with a pH 7 and pH 9 elution using a smaller bed volume of 2.8cm x 3.6cm and equilibrated in1.0M pH 7 Tris followed by 10 mM Tris. 2ml aqueous extract was loaded onto the column, which was eluted with 10mM pH 7 Tris collecting fifty 3ml fractions (elution volume 0-150ml) followed by a pH 9 Tris elution (elution volume 151-510ml) and then a high salt elution with 3.0M $MgCl_2$ pH 9.0 Tris (elution volume 513-630). Fractions corresponding to each of the two peaks were pooled and assayed by the well method.

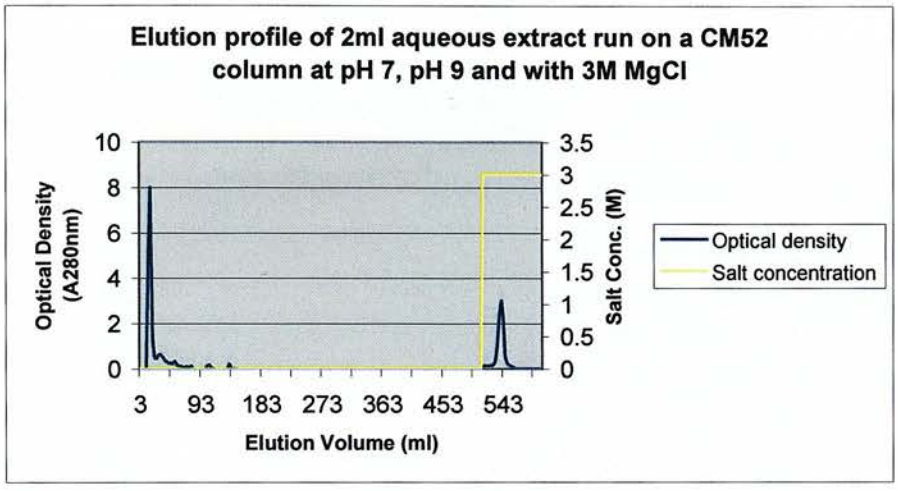


Figure 4.13 Elution profile of 2ml aqueous extract run on a CM52 column with a 2.0M NaCl pH 6.0 equilibration followed by 3.0M $MgCl_2$ pH 9.0 elution.

Elution volume	Zones of Inhibition
PH 7 elution (first peak)	0mm
3.0M $MgCl_2$ elution (second peak)	16mm
Control, equal volume of elution buffer concentrated in the same way	0mm

Table 4.4 Active fractions from CM52 chromatography

The results show a combination of salt elution and pH 9 running buffer is necessary to elute the active principle(s) from the column. The experiment was repeated with a salt gradient to allow a more gradual elution of active principle(s) and all at pH 9.0 to see if the method could be simplified.

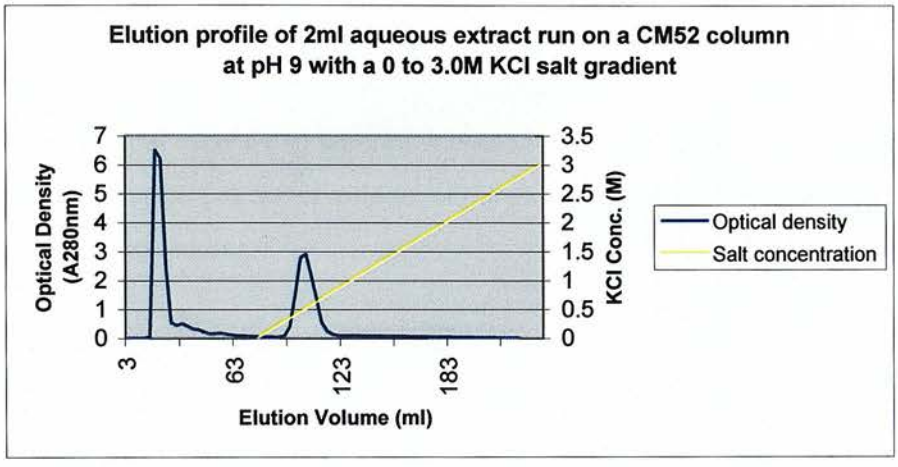


Figure 4.14 Elution profile of 2ml aqueous extract run on CM52 column with a 2.0M NaCl pH 6.0 equilibration followed by a 0 to 3.0M KCl pH 9.0 salt gradient.

Elution volume	Zones of Inhibition
Flow through (1-60ml)	0
Salt gradient (96ml)	7mm
Salt gradient (99ml)	10mm
Salt gradient (102ml)	11mm
Positive control	8mm

Table 4.5 Active fractions from CM52 chromatography

Two main peaks were resolved and activity was released from the column suggesting CM52 ion exchange as another potential step in the purification of the active principles.

4.4 Activity-guided fractionation of the aqueous bark extract

The three successful chromatography methods (gel filtration, anion and cation exchange) were then put together to see if they could be used to isolate the active principle(s) from the crude extract. A larger volume of the extract was used to ensure a high yield of active compound(s).

4.4.1 Extraction and phase separation

25g dried *Lunasia* bark was ground to a fine powder in a coffee grinder and then extracted with 500ml ethanol stirring magnetically for 30 minutes. The extract was then filtered through Whatman No 1 filter paper and dried down in a rotary evaporator. The resulting residue was taken up in 100ml water and defatted with 300ml chloroform stirring thoroughly for 10 minutes. The aqueous phase was then freeze-dried and taken up in 5ml water to form a highly concentrated extract.

4.4.2 Anion exchange chromatography

A Dowex column (bed size 3cm x 2.4cm) was set up and equilibrated with 10mM pH 7.0 Tris. The extract was eluted through the column with the same buffer collecting twenty 5ml fractions. Each fraction was then assayed by the disk method.

Fraction	Zones of inhibition (mm)
1	10
2	15
3-20	0

Table 4.6 Active fractions from dowex column.

Fractions 1 and 2 were combined, freeze-dried and taken up in 5ml ethanol.

4.4.3 LH-20 Gel filtration

A Sephadex LH-20 column(bed size 60cm x 3.2cm) was set up and equilibrated in ethanol. The 5ml sample was loaded into the column and eluted with ethanol collecting eighty 5ml fractions. Absorbance readings were taken by diluting 5µl of each fraction in 950µl water and vortexing thoroughly. Fractions were assayed by the disk method.

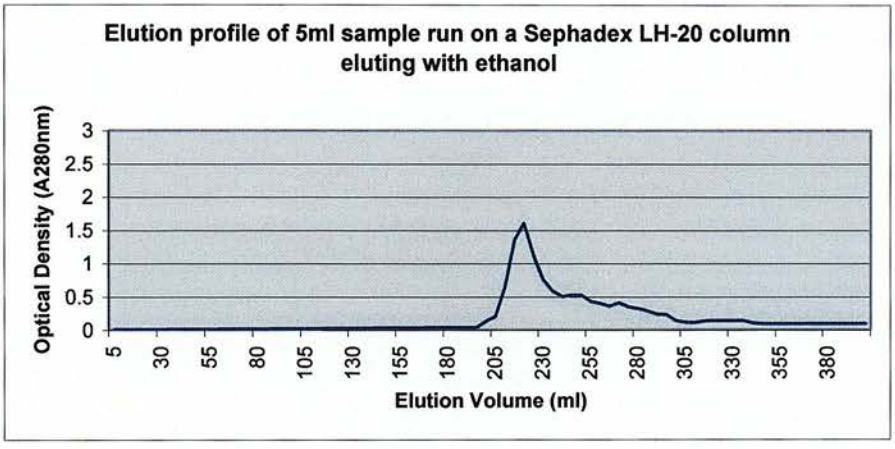


Figure 4.15 Separation of the dowex anion exchange fractions on Sephadex LH-20

Fraction	Elution volume (ml)	Zones of inhibition
40	200	0
41	205	10
42	210	10
43	215	9
44	220	8
45	225	7
46-55	230	0

Table 4.7 Active fractions from the Sephadex LH-20 column.

Fractions 41-45 were dried down with a rotary evaporator and taken up in 5ml water.

4.4.4. Cation exchange chromatography

A CM52 column was set up (bed size 9.5cm x 2.8cm) and equilibrated in pH 7.0 10mM Tris. The 5ml sample was loaded into the column and eluted on a flow-through with 10mM pH 7.0 Tris for twenty 5ml fractions. The column was then eluted with a 10mM pH 9.0 Tris 0.0M KCl to 3.0M KCl salt gradient collecting sixty 3ml fractions.

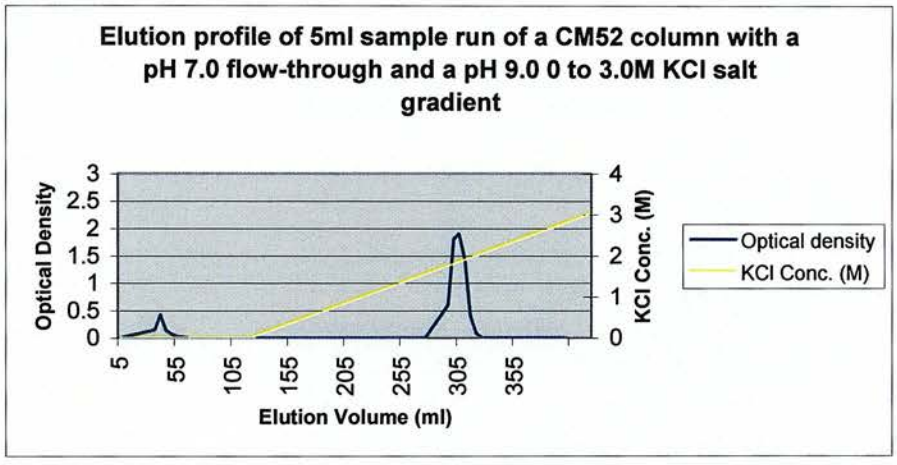


Figure 4.16 Separation of the gel filtration active fractions on a CM52 column

Fraction	Elution Volume (ml)	Zones of Inhibition (mm)
7	35	0
8	40	0
9	45	0
59	295	7
60	300	13
61	305	14
62	310	11
63	315	7
64	320	0

Table 4.8 Active fractions from the CM-52 column

Fractions 59-63 were freeze-dried and taken up in 30ml ethanol/water 1:1 as the sample would not dissolve in pure ethanol and would not dissolve at all in 5ml water or ethanol due to the salt.

4.4.5. Desalting

The LH-20 column was washed through with 500ml ethanol and the 30ml sample loaded into the column and eluted as before collecting eighty 5ml fractions.

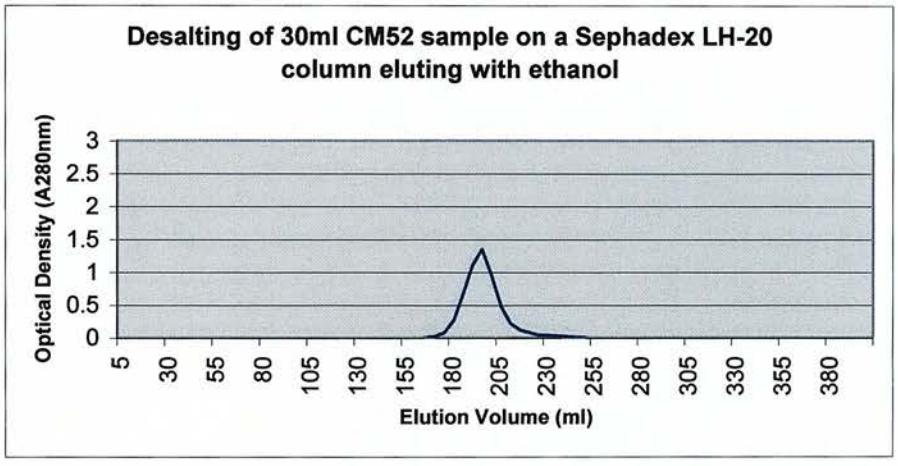


Figure 4.17 Desalting of the CM52 active fractions on Sephadex LH-20.

Fraction	Elution Volume (ml)	Zones of Inhibition (mm)
36	180	0
37	185	7
38	190	7
39	195	8
40	200	7
41	210	0

Table 4.9 Active fractions from desalting step.

Fractions 37-40 were combined, dried down and taken up in 1ml water.

4.4.6. High performance liquid chromatography

200µl of the sample were run on an Applied Biosystems 130A HPLC using a 5µ 4.6mm x 250mm C-18 column and eluting on a half hour gradient from 20-100% solvent B where solvent A was 0.1% TFA and solvent B 70% MeCN 0.08% TFA. The flow rate was set at 500µl/min and the chart plotter sensitivity 100mv and chart plotter speed 2mm/min.

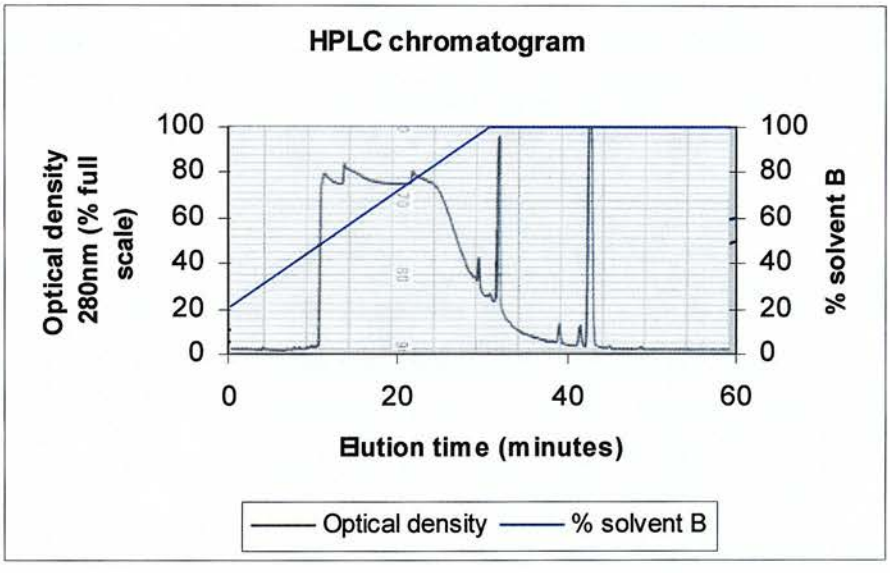


Figure 4.18 Separation of pooled active fractions from open column purification steps using HPLC.

The trace shows the presence of several different compounds so the remainder of medium pressure chromatography sample was run under the same conditions 200µl at a time collecting 10 fractions per run using the collect by time function on the fraction collector. Each run gave a trace identical to that above. 50µl of each fraction was assayed in wells as the disk method using 5µl gave no zones of inhibition.

Fraction	Zones of inhibition (mm)
1-3	0
4 (24 mins)	21
5-10	0

Table 4.10 Active fractions from HPLC separation.

Fraction 4 which corresponded to an elution time of 24 minutes was freeze-dried and taken up in 2ml water but would not dissolve fully. The sample was freeze-dried a second time and then taken up in 950µl water to which concentrated ammonia was added 5µl at a time to see if raising the pH which would have been lowered due to concentration of TFA in the sample would help increase solubility. After adding 30µl ammonia, the insoluble material had completely dissolved and the final volume was adjusted to 1ml with water.

The 1ml sample was then run on the HPLC 200µl per run, eluting isocratically at 30, 40, 50 and 100% B respectively using the same C-18 column and solvents as before. However, it was found that the excessive tailing on peak 1 could be significantly reduced by temporarily increasing the % solvent B up to 50 and then back down to 30 without eluting the other peaks and as this helped to reduce run time it was carried out on subsequent runs.

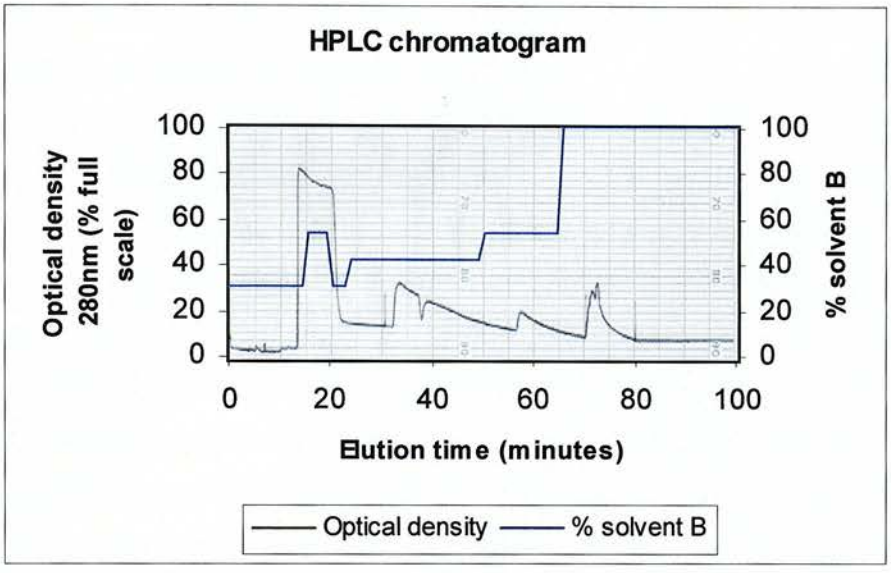


Figure 4.19 HPLC active fractions re-separated on a C-18 column.

Each of the four peaks were freeze-dried and taken up in 5ml distilled water. 5µl of each peak was assayed by the disk method. Only peak 1 was active and this was freeze-dried to form 66.7mg of a white powder.

Peak	Zones of inhibition (mm)
1	13
2	0
3	0
4	0

Table 4.11 Active fractions from second HPLC separation.

4.5 Structure determination of sample

The sample was sent to Dr I. Sadler at the Ultra-High Field NMR Centre, Department of Chemistry, University of Edinburgh who used a combination of H^1, C^{13} NMR and mass spectroscopy to gain an unequivocal characterisation of the highly pure single compound

present in the sample. NMR spectra were recorded using a Varian INOVA 600MHz spectrophotometer.

Analysis of the NMR and mass spectra of the sample showed it to be a 2'-*O*-trifluoroacetyl derivative of lunacridine (MW = 401) (Fig.4.20). The mass spectrum shows a molecular ion at 401 (exact measurement 401.145) and fragment ions at 304 (loss of CF_3CO), 273 (loss of CF_3CO and CH_3O) and 69 (CF_3) consistent with the molecular formula $C_{19}H_{23}F_3NO_5$ (410.145). The 1D proton spectrum obtained at 360 MHz using d_4-methanol as solvent was well resolved and showed the presence of the following fragments: a 1,2,3-trisubstituted benzene ring, a 3-methylbutyl group with a single bonded oxygen at C-2 and three methyl groups directly bonded to oxygen or nitrogen.

The carbon 13 NMR spectrum showed 19 signals: 2 CH_3 groups attached to carbon, 2 CH_3 attached to oxygen, 1 CH_3 attached to nitrogen, one aliphatic CH_2 group, one aliphatic CH bonded to oxygen, one aliphatic CH bonded to oxygen, three aromatic CH groups, 6 aromatic/carbonyl quaternary carbons and two signals from a CF_3CO group showing coupling to fluorine (the weak signal from the CF_3 group was only visible in a spectrum from a more concentrated solution).

Given the source of the material and its molecular formula, a lunacridine derivative seemed likely and analysis of the 2-dimensional 1-bond (HMBC) and multiple-bond (HMBC) proton-carbon correlation spectra confirmed this, and allowed unambiguous assignment of all the proton and carbon signals. The 1-bond (HMBC) correlation spectrum allowed the assignment of the protonated carbons except that distinction between the two methoxy signals and the H/C-5 and H/C-7 signals was not possible at this stage. The multiple bond (HMBC) spectrum resolved these assignments and also allowed assignment of the non protonated carbons.

The carbonyl group (C-2) was assigned by correlation with the N-methyl protons (the carbon shifts of the other correlations being too low). The methoxy proton signals at 4.14d and 4.58d correlate respectively with the carbon signals at 150.4d and 162.4d. The latter proton signal, but not the former, also correlates with C-2'. Thus the latter corresponds to the 4-methoxy group and thus C-4 and C-8 are both assigned. This is confirmed by the correlation of C-4 (but not C-8) with both H-1' signals and by the correlation of C-8 (but not C-4) with the H-6 and the signal at 7.63d, which must therefore be H-7 (and not H-5). C-8a is identified by correlation with the N-methyl protons, not shown by the remaining quaternary at 126.7d which must therefore be C-4a. C-4a assignment is confirmed by its correlation with H-5 and H-6. Other 2- and 3- bond correlations are listed in the table and all are consistent with the proposed structure.

The trifluoroacetyl resonance assignments follow from the magnitude of the C-F couplings. Since the parent molecule has a hydroxyl group at C-2' this must be the site of trifluoroacetylation. For comparison purposes, literature values for the carbon-13 chemical shifts (obtained using $CDCl_3$ as solvent) (Brown, 1980) of the non-trifluoroacetylated lunacridine are given in parentheses in the table. Except for C-2', the agreement is excellent. The 15 ppm increase in chemical shift of C-2' is expected for *O*-trifluoroactylation.

Site	δ_C	δc	δ_H	proton	J_{HH} (Hz)	Proton - Carbon
	Lit*		multiplicity		long-range correlations	
		parent $CDCl_3$			from HMBC spectrum	
2	166.8	(167.1)	-			
3	103.0	§	-			
4	162.4	(161.3)	-			

4a	121.4	(120.3)	-			
5	115.2	(116.1)	7.98	(dd)		8.1, 1.4 C-4, C-4a, C-7, C-8a
6	127.4	(123.0)	7.70	(t)	8.1	C-4a, C-5, C-7, C-8
7	115.0	(113.9)	7.63	(dd)		8.1, 1.4 C-5, C-8, C-8a
8	150.4	(149.1)	-			
8a	126.7	§	-			
1'a	30.7	(30.0)	3.78	(dd)		15.8, 7.9 C-2, C-3, C-4, C-4a (w), C-2', C-3'
1'b			4.17	(dd)		15.8, 7.9 C-2, C-3, C-4, C-4a (w,) C-2', C-3'
2'	93.2	(77.4)	5.21	(dt)	7.9, 6.8	C-2, C-3, C-3', C-4', C-5'
3'	32.3	(34.8)	2.27	(oc)		6.8 C-1', C-2', C-4', C-5'
4'	16.1	(18.5)	1.25	(d)	6.8	C-2', C-3', C-5'
5'	15.7	(17.7)	1.19	(d)	6.8	C-2', C-3', C-4'
N-Me	38.4	(35.8)	4.41	(s)		C-2, C-3, C-8a
4-O-Me	58.5	(62.0)	4.58	(s)		C-4, C-1', C-2'
8-O-Me	55.4	(56.7)	4.14	(s)		C-8
$\underline{C}F_3$	113.0 (q)		[$^1J_{CF}$ = 284 Hz]			
$\underline{C}OCF_3$	159.6 (q)		[$^2J_{CF}$ = 37 Hz]			

d = doublet, dd = doublet of doublets, dt = doublet of triplets, q = quartet, oc = octet, s = singlet, t = triplet

§ = signal not reported w = weak signal

Table 4.12 ^{1}H and ^{13}C NMR Spectral Data of 2'-*O*-Trifluoroacetyl-lunacridine in CD_3OD

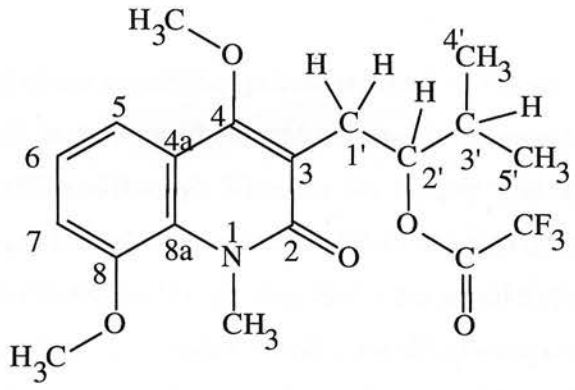


Figure 4.20 Structure of 2'-*O*-Trifluoroacetyl-lunacridine.

The compound is therefore the trifluoroacetyl derivative of lunacridine, trifluoroacetylation most likely having occurred during or after the HPLC step since TFA was used as a mobile phase modifier.

4.6 Purification by HPLC

The isolation of Lunacridine by open column chromatography used numerous steps each of which increased the chances of active principles undergoing chemical changes and despite the range of separation media used HPLC was still required as a final step in the purification. A second disadvantage of using a multistep protocol is an increased chance of loosing active principles which may not be noticed if the lost compound is one of several that contribute towards the total activity of the crude extract. Thus it was not known whether the Lunacridine was the sole antibacterial ingredient in the crude extract or if there were others that had been lost or degraded during one of the purification steps and further more a trifluoroacetyl derivative rather than native lunacridine had been isolated. It was therefore decided to develop a simplified two-stage protocol based on an initial cleanup step such as phase extraction followed by a single well resolved HPLC separation.

4.6.1 Developing an initial cleanup step

A variety of phase extraction systems were trialled to see if the activity of the crude extract could be partitioned into chloroform. 5g *Lunasia* bark was extracted with 100ml ethanol and filtered through Whatman No.1 paper. The ethanolic extract was evaporated to dryness and the residue taken up in 50 ml water to make a standardised extract. 0.1ml of various solvents, acids and bases then were added to 0.9 ml aliquots of the extract and the mixture vortexed thoroughly with 2 ml chloroform to allow partition of the active principles. In each case the optical absorbance at 280nm for the two different phases was recorded and 100µl samples of each assayed in wells.

Tube	Extract volume (ml)	Additives (0.1ml)	$CHCl_3$ volume	Abs 280nm organic layer	Abs 280nm aqueous layer	ZOI Aq layer	ZOI Organic layer
1	0.9	Water	2	0.402	0.540	14	0
2	0.9	Methanol	2	0.399	0.503	14	0
3	0.9	Ethanol	2	0.437	0.506	15	0
4	0.9	Butanol	2	0.531	0.531	16	0
5	0.9	Butan-2-one	2	0.517	0.517	15	0
6	0.9	Acetonitrile	2	0.495	0.575	16	0
7	0.9	Methanal	2	0.463	0.563	16	0
8	0.9	10%HCl	2	0.651	0.483	14	13
9	0.9	10% NH_3	2	0.556	0.728	11	0
10	1.0	Made to 1M KCl	2 x3	0.113	0.669	0	15.5

Table 4.13 Phase extraction methods applied to the bark extract. In each case, UV absorbance at 280nm were recorded for both the organic and aqueous layers along with zones of inhibition produced by assaying 100µl of each layer.

Table 4.13 shows KCl to be the most effective additive since it partitions all the activity of the bark extract into the chloroform layer and the absorbance readings suggest a large amount of impurities are left behind in the aqueous layer. The method was further

improved by back extracting the active principles into water as follows. At each stage 100µl of the aqueous sample were assayed in wells to check for loss of activity.

Step	Sample/Purification step	ZOI (mm)
1	5g finely ground *Lunasia* bark extracted with 100ml water and filtered through Whatman No 1 paper.	18
2	Extract made to 1M KCl	17
3	Aqueous phase extracted thoroughly with 100ml chloroform and the chloroform layer removed with a separating funnel	13
4	Aqueous layer re-extracted with a further 100ml chloroform	9
5	Aqueous layer re-extracted a third time with 100ml chloroform	0
6	Chloroform layers combined and back extracted with 100ml water vortexing thoroughly.	15
7	Aqueous layer removed and freeze-dried; the resulting powder was then taken up in 5ml water.	N.A.
	Re-extracted bark	8

Table 4.14 Optimised phase extraction method for *L. amara* bark extract. At each step 100µl samples were assayed in wells to check for loss of activity.

Comparison of the phase extracted sample with an aqueous extract reveals 79% of the bark material (by mass) had been removed through the phase extraction process (Table 4.15). Although water is a less selective extraction solvent than ethanol it was included in the extraction method to avoid an extra concentration step since ethanol is miscible with chloroform.

Sample	Solids present after freeze-dying or rotary evaporation (grams)
5g bark extracted with 100ml water	0.72
5g bark extracted with 100ml ethanol	0.55
Phase extracted sample	0.15

Table 4.15 Comparison of the amounts of dried solids present in ethanolic, aqueous and phase extracted *L. amara* bark extracts after concentration to dryness.

4.6.2 HPLC optimisation

HPLC is a technique that has developed from classical silica column chromatography to meet the demands of more difficult separations in which analytes may differ by only one functional group (Robards, 1994). The increased performance over open column techniques is achieved through the use of column packings with reduced particle size (3-5 microns in diameter) which allow increased mass transfer between mobile and stationary phases. The small particle size also minimizes the effect of eddy diffusion that arises as a result of the differing flow paths an analyte molecule may take to pass around stationary phase particles. By reducing flow multipaths, zone broadening (the widening of analyte bands in the column due to diffusion) is minimised and the separation efficiency improved (Robards, 1994).

Initial separation of the *L. amara* bark extract was carried out on a C-18 column using a mobile phase containing trifluoroacetic acid which is commonly used in the analysis of peptides. The resulting peak shape was very poor (Figure 4.19) displaying a high degree of peak tailing. Such distortion of the ideal Gaussian-shaped peak towards a Langmuir isotherm is common in systems where secondary interaction sites are present or where the interaction between analyte molecules is weak relative to their interaction with the stationary phase (Robards, 1994). In such systems the areas of highest analyte concentration in the centre of the band have least opportunity of interaction with the stationary phase and so migrate fastest, yielding a tailed peak shape which is dense at the front and progressively thinner towards the tailing edge (Robards, 1994). The peak tailing effect produces broader peaks which results in loss of resolution. The secondary interaction sites are likely to be free silanol hydroxyl groups that are present in even the most intensively derivitised reverse-phase columns where silica is used as the support for the bonded phase. Compounding this problem is the fact that quinolones and quinolines are notorious as a class of compounds for secondary interactions with stationary phase silanol groups (Kim et al., 2002).

A large number of HPLC runs were carried out using a wide array of conditions in which both stationary, mobile phase and run temperature were varied in an attempt to reduce this effect. For convenience only a small selection of separations are represented here. Over the course of this optimisation process several HPLC methods yielding upright peaks with near baseline separation and minimal peak tailing were developed. For each of these methods the phase extracted sample was run several times at overload conditions to prepare sufficient quantities of compounds for analysis by NMR. Unless stated otherwise the flow rate was always set at 1.5ml/minute and the injection volume was 5µl. The detector wavelength was set at 280nm with optical density read as detector mV output and the column temperature was left as ambient. In some HPLC runs the anti-oxidant β-mercapto ethanol was included in the mobile phase as a precaution against oxidation of active principle(s).

To begin with, the phase extracted sample was run with standard reverse-phase conditions on a C-18 column using water and acetonitrile as solvents A and B respectively.

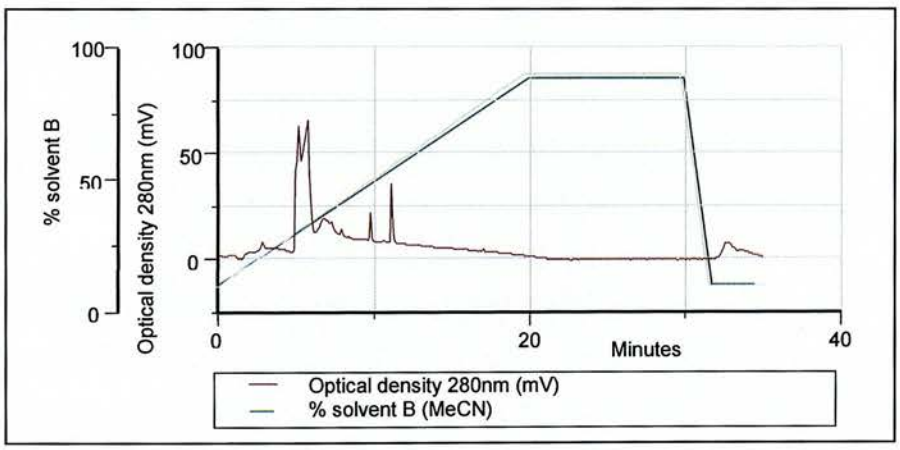


Figure 4.21 Separation on a Thermohypersil beta basic C-18 5µ 4.6mm x 150mm column with solvent A 5mM β-mercaptoethanol and solvent B 5mM β-mercaptoethanol MeCN.

The standard conditions yielded crowded peaks with some peak tailing and progressively shallower gradients failed to improve the resolution. Peak tailing is often caused by interactions between free (un-capped) silanol groups in the non-bonded phase of the column and positive charges on analyte molecules so a polymeric non-silica column was used to see if this would reduce the peak tailing.

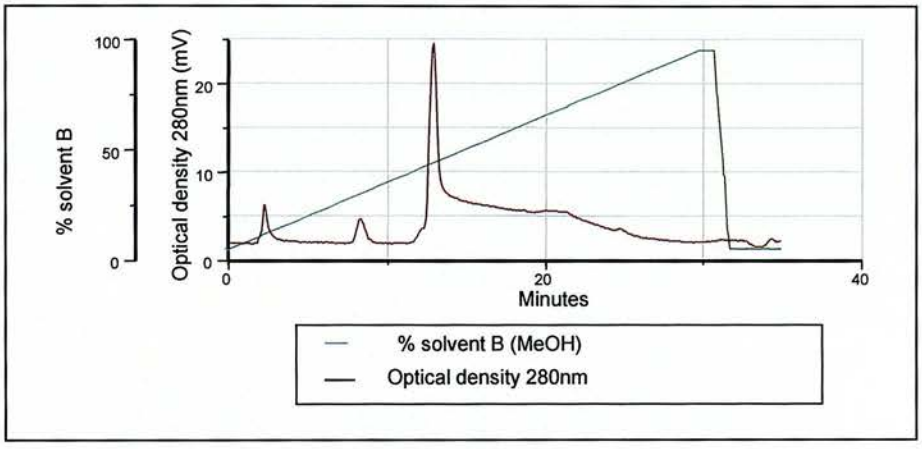


Figure 4.22 Separation on a Polymeric TPR-100 4.6mm x150mm column with solvent A H_2O and solvent B Methanol.

The polymeric column gave little improvement over the C-18 column suggesting the peak tailing effect may not be due to secondary interactions with the stationary phase.

Next, a range of ion suppression mobile phases each buffered to a different pH were used to determine if suppression of analyte positive charge could result in an improved separation. The use of strongly basic mobile phases was only possible with the polymeric stationary phase so this column was used throughout.

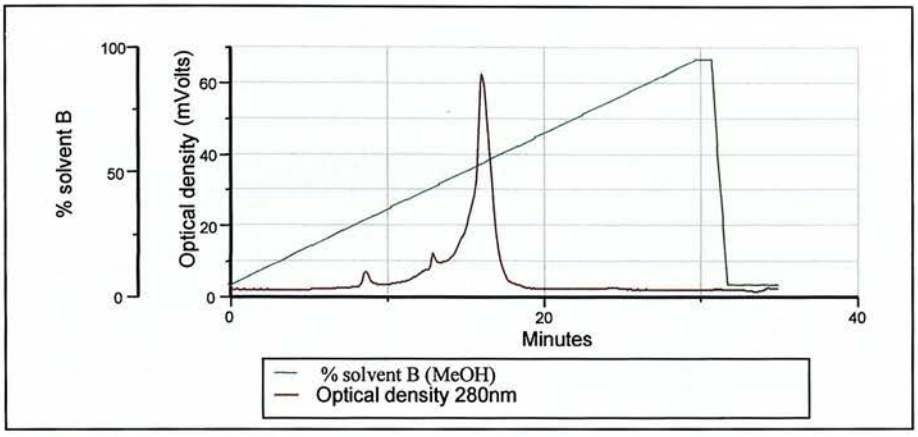


Figure 4.23 Separation on a polymeric TPR-100 4.6mm x150mm column with solvent A 10mM HCl KCl buffer pH 1.0 and solvent B as Methanol.

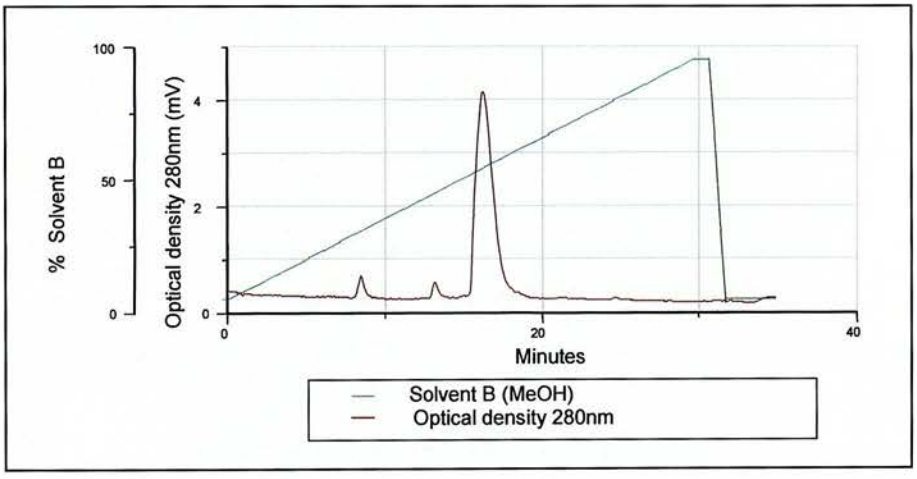


Figure 4.24 Separation on a Polymeric TPR-100 4.6mm x150mm column with solvent A as 10mM citrate pH 3.0 and solvent B Methanol.

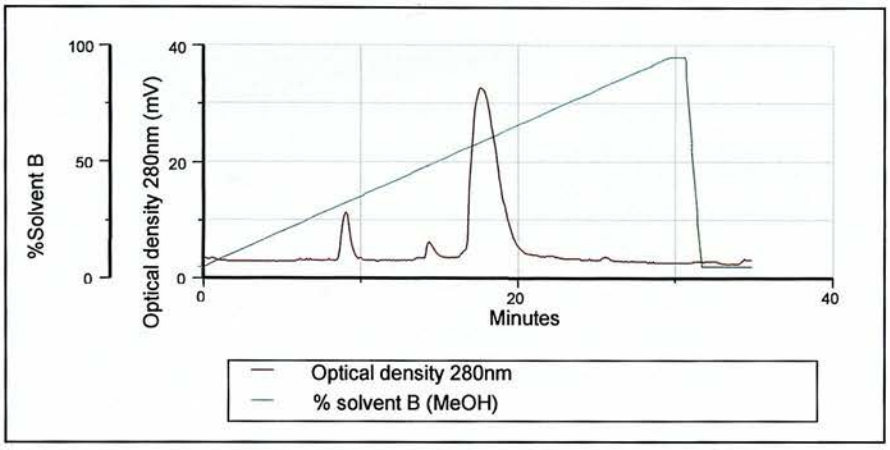


Figure 4.25 Separation on a Polymeric TPR-100 4.6mm x150mm column with solvent A as 10mM citrate pH 5.0 and solvent B Methanol

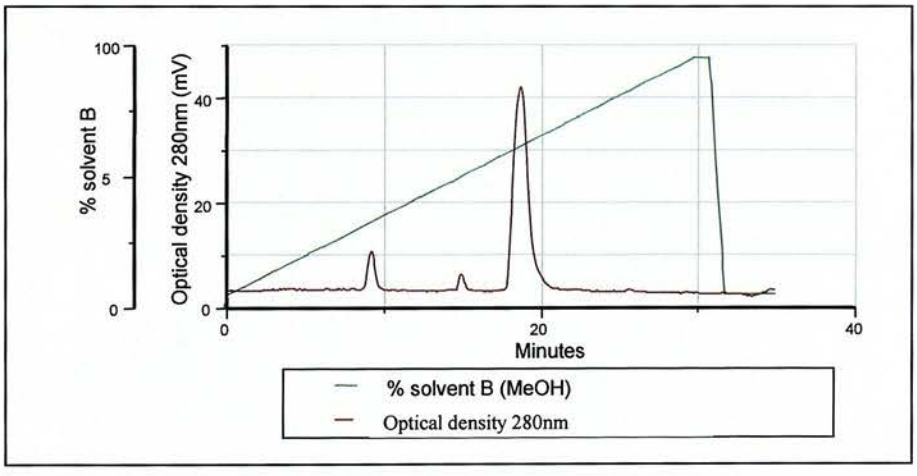


Figure 4.26 Separation on a Polymeric TPR-100 4.6mm x150mm column with solvent A as 10mM HCl Tris pH 7.0 and solvent B Methanol.

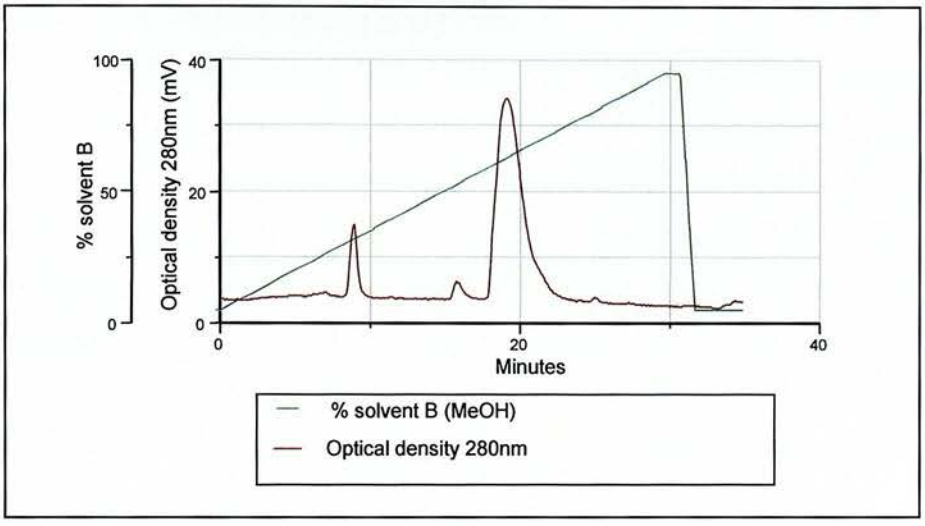


Figure 4.27 Separation on a Polymeric TPR-100 4.6mm x150mm column with solvent A as 10mM HCl Tris pH 9.0 and solvent B as methanol.

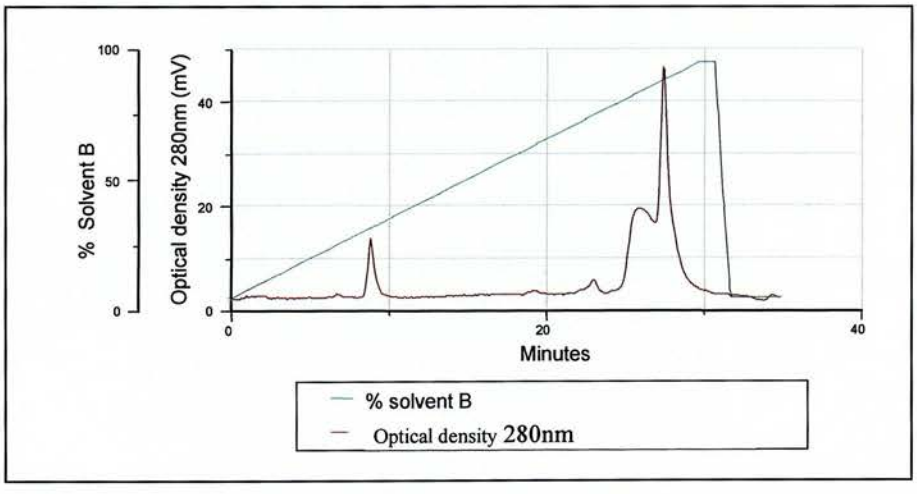


Figure 4.28 Separation on a Polymeric TPR-100 4.6mm x150mm column with solvent A as10mM $Na_2CO_3/NaHCO_3$ buffer pH 10.6 and solvent B as methanol.

The results show that increasing the pH of the mobile phase increases the retention times of the analytes presumably due to ion suppression. Thus at pH 10.6 the polarity of the analytes is reduced compared to pH 1 (resulting in increased affinity for the stationary phase). The resolution of the separations are still poor however with broad peaks and with some peaks not resolved at all.

Further separations were carried out with basic mobile phases using a C-18 bonded silica column to see if the same peak tailing would occur. The mobile phases were not buffered as the column would not tolerate strongly basic mobile phases.

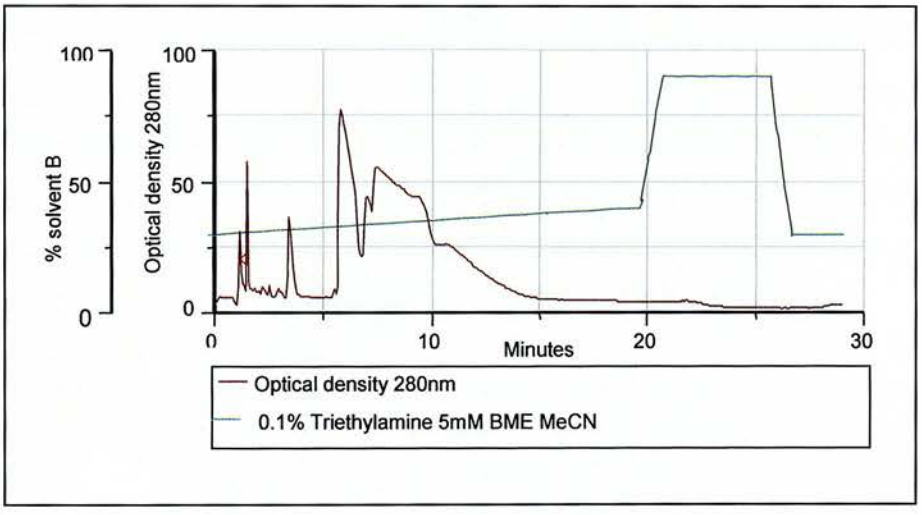


Figure 4.29 Separation on a Thermohypersil beta basic C-184.6mm x 150mm with solvent A as 0.1% triethylamine 5mM BME and solvent B as 0.1% triethylamine 5mM BME MeCN.

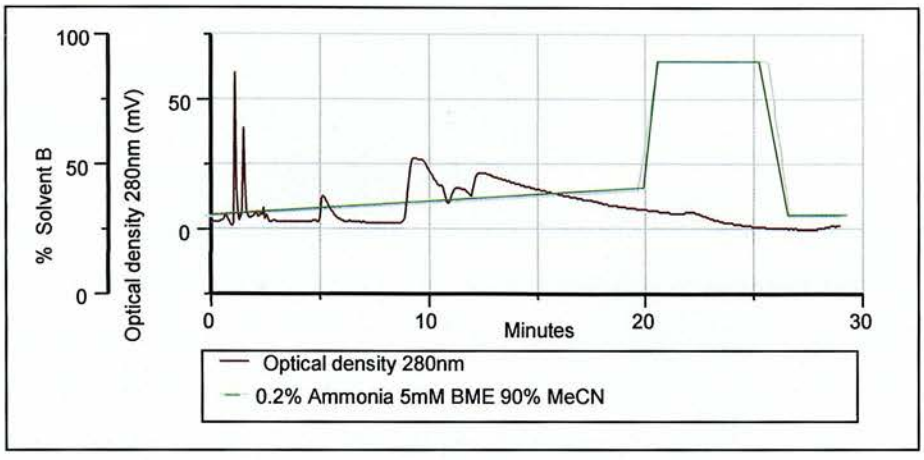


Figure 4.30 Separation on a Thermohypersil beta basic C-184.6mm x 150mm with solvent A as 0.2% ammonia 5mM BME and solvent B as 0.2% ammonia 5mM BME 90% MeCN

The results show that increasing mobile phase pH results in more pronounced peak tailing than that seen with the polymeric column. Therefore basic mobile phases were not used in future separations. Since the CM52 ion exchange experiments indicate that lunacridine is positively charged, a negatively charged ion pair heptane sulphonic acid was used to see if this would interact with the positive charge and allow a purely reverse-phase separation without any secondary interactions.

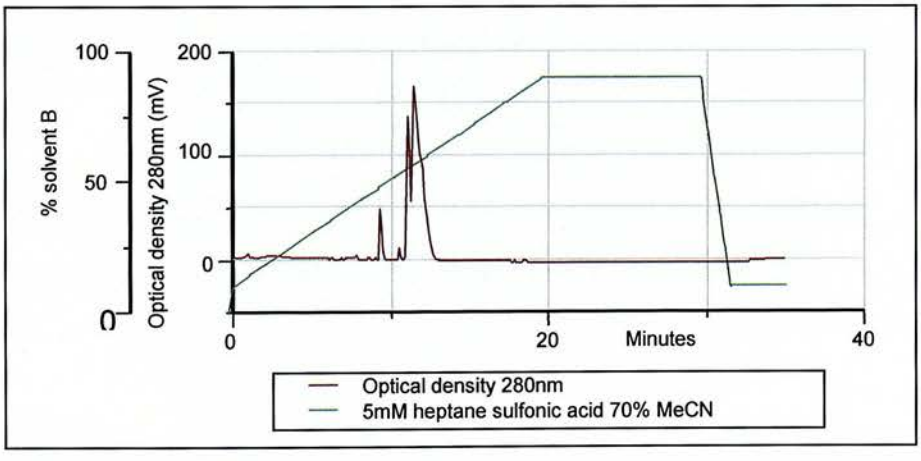


Figure 4.31 Separation on a Thermohypersil beta basic C-184.6mm x 150mm column with solvent A as 5mM heptane sulphonic acid and solvent B as 5mM heptane sulphonic acid 70% MeCN.

The peaks are two closely grouped to allow them to be collected easily and changes in the gradient did not improve the resolution.

Lunacridine is a quinoline and is therefore structurally related to the quinolone antibiotics. Numerous methods exist for the detection of antibacterial quinolones from blood plasma and the predominant feature of such methods is the use of phosphate mobile phases (Gertz, 1990). Various phosphate based mobile phases were therefore tested to see if they would produce the desired improvement in peak shape.

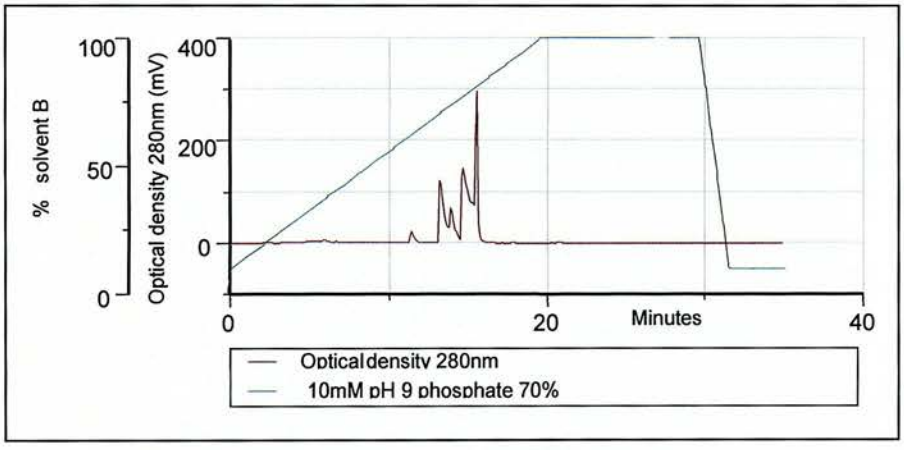


Figure 4.32 Separation on a Thermohypersil beta basic C-184.6mm x 150mm with solvent A as 10mM pH 9 potassium phosphate and solvent B as 10mM pH 9 potassium phosphate 70% MeOH.

The run was repeated at pH 8 with a shallower gradient of 40% to 60% solvent B resulting in a marked improvement in the quality of the separation.

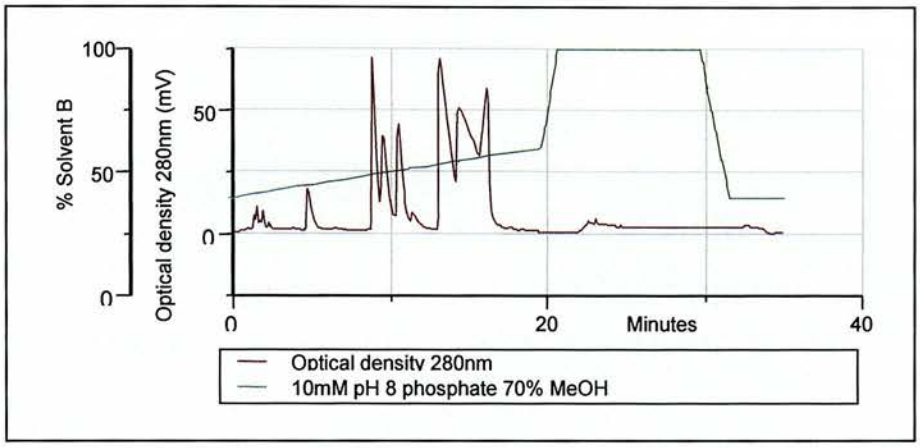


Figure 4.33 Separation on a Thermohypersil beta basic C-184.6mm x 150mm with solvent A as 10mM pH 8 potassium phosphate and solvent B as 10mM pH 8 potassium phosphate 70% MeOH.

Next, a more acidic phosphate mobile phase using phosphoric acid was tried but this further increased the peak tailing effect.

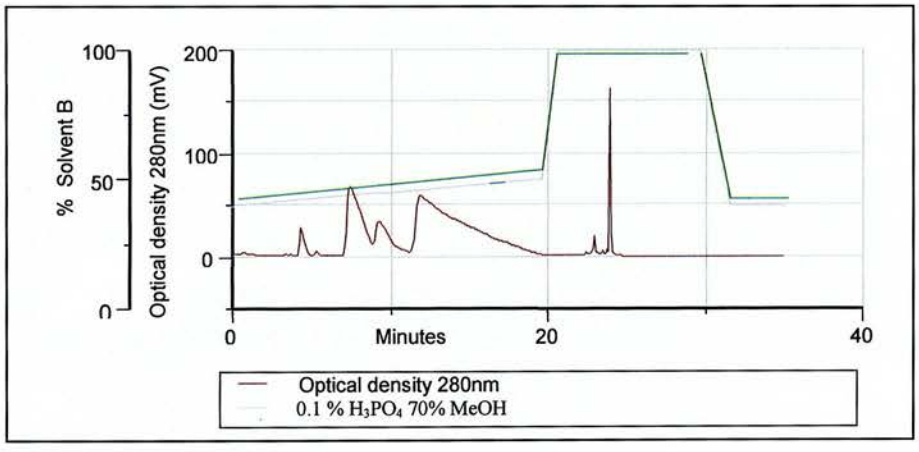


Figure 4.34 Separation on a Thermohypersil beta basic C-18 4.6mm x 150mm with solvent A as 0.1% H_3PO_4 and solvent B 0.1% H_3PO_4 70% MeOH

Increasing solvent B at 11minutes revealed the highly distorted peak eluting at 12 minutes to be composed of two separate peaks. The run was repeated again with a sudden increase to 70% B to try and further resolve them.

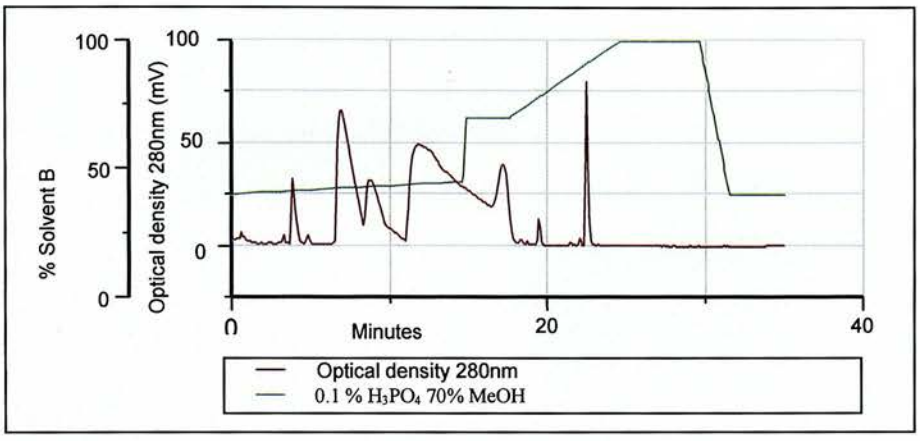


Figure 4.35 Separation on a Thermohypersil beta basic C-18 4.6mm x 150mm with solvent A as 0.1% H_3PO_4 and solvent B 0.1% H_3PO_4 70% MeOH

The use of phosphate mobile phases improved the quality of the separation but it was still far from being the baseline resolution that would allow a definitive analysis of the active principles in the bark extract. A new column sold under the brand name Hypercarb which is based on a porous graphite stationary phase was used to see if its novel chemistry would mitigate the effects of peak tailing encountered with basic analytes on a silica bonded phase. The graphite stationary phase sorbs polar analytes via a polar retention effect but is equally capable of reverse phase interactions. It is also able to withstand high temperatures which increase the partition rate of analytes between the mobile and stationary phases (Knox, 1986). The column was eluted with a mobile phase containing 1:1 IPA/0.1% TFA MeCN as recommended by the manufacturers.

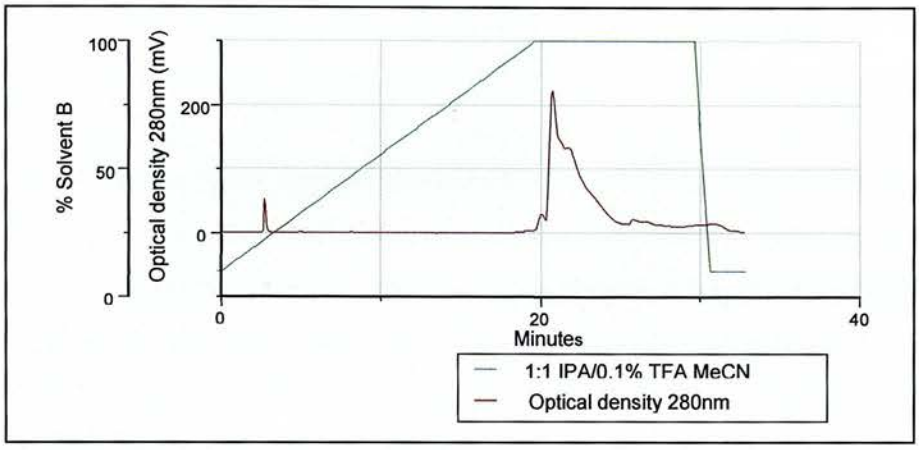


Figure 4.36 Separation on a Hypercarb 5μ 4.6 x 50mm with solvent A as 0.1% TFA and solvent B B 1:1 IPA/0.1% TFA MeCN.

The run was repeated placing the column in a water bath at 70°C.

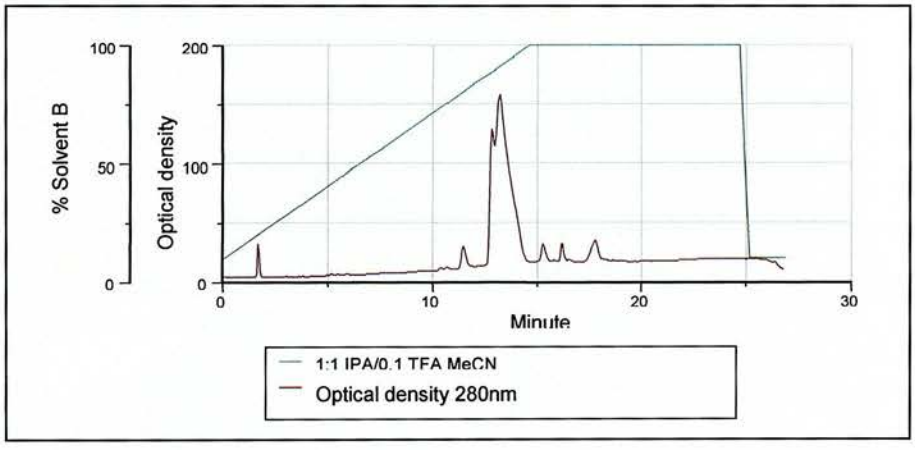


Figure 4.37 Separation on a Hypercarb 5μ 4.6x50mm with solvent A as 0.1% TFA and solvent B 1:1 IPA/0.1% TFA MeCN with a column temperature of 70°C.

Neither increase in column temperature or variation in solvent gradients were able to give a satisfactory separation.

Further research revealed the phenyl-hexyl stationary phase as a good candidate to try next. The phenyl-hexyl phase is said to give increased selectivity for polar heterocycles; the phenyl group promoting pi-pi interactions with aromatic analyte molecules and the hexyl chain providing a reverse-phase character (Marchand et al., 2005). Standard mobile phases containing methanol or acetonitrile failed to elute much material from the column confirming the presence of a different mode of interaction from the columns used so far. The H_3PO_4 mobile phase (below) provided improved peak relative to the C-18 column but as with other runs the main peaks were merged and this was not improved with shallower gradients.

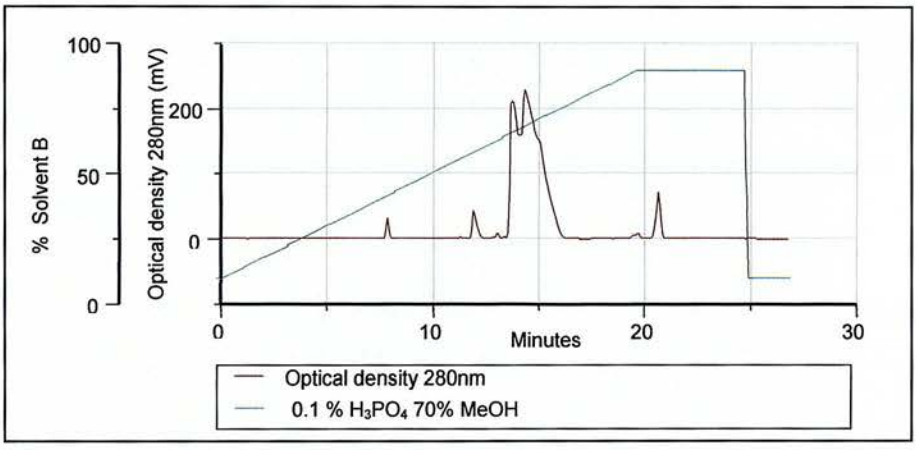


Figure 4.38 Separation on a Phenyl-Hexyl 4.6mm x 150mm with solvent A as 0.1% H_3PO_4 and solvent B 0.1% H_3PO_4 70% MeOH.

The phenyl/hexyl column was run with the mobile phase recommended for the hypercarb column to see if the presence of isopropanol which is higher up the elutrophic series than either methanol or acetonitrile would improve the resolution. The resolution showed some improvement and by using a shallower gradient a chromatogram was produced that showed basline resolution of seven peaks. Removing TFA from the mobile phase was found to reduce the quality of the separation as was reducing the level of isopropanol.

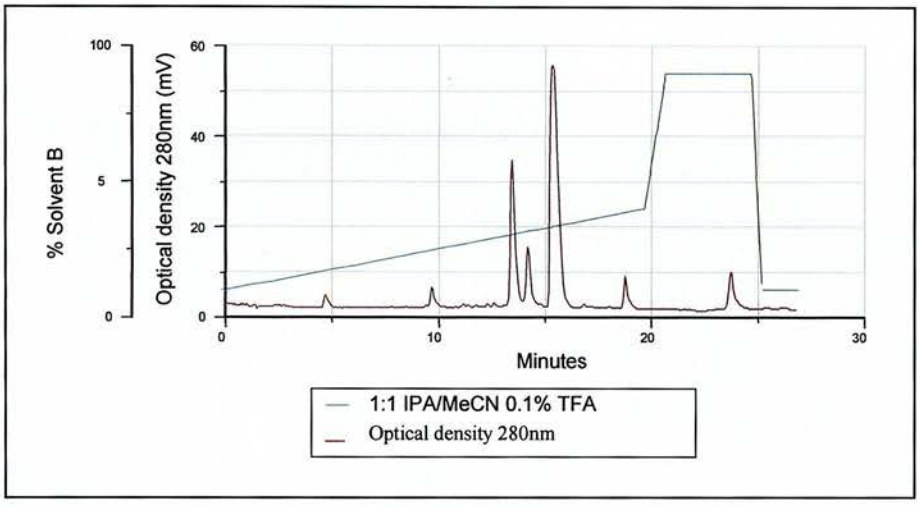


Figure 4.39 Separation on a Phenyl-Hexyl 4.6mm x 150mm column with solvent A as 0.1% TFA and solvent as B1:1 IPA/0.1% TFA MeCN injecting 5µl of a 1/10 diluted sample. Column temperature set at ambient. The combination of TFA, isopropanol and a shallower solvent gradient result in a markedly improved separation.

The run was repeated replacing TFA with different acidic mobile phase modifiers to determine if other acids would give the same degree of resolution.

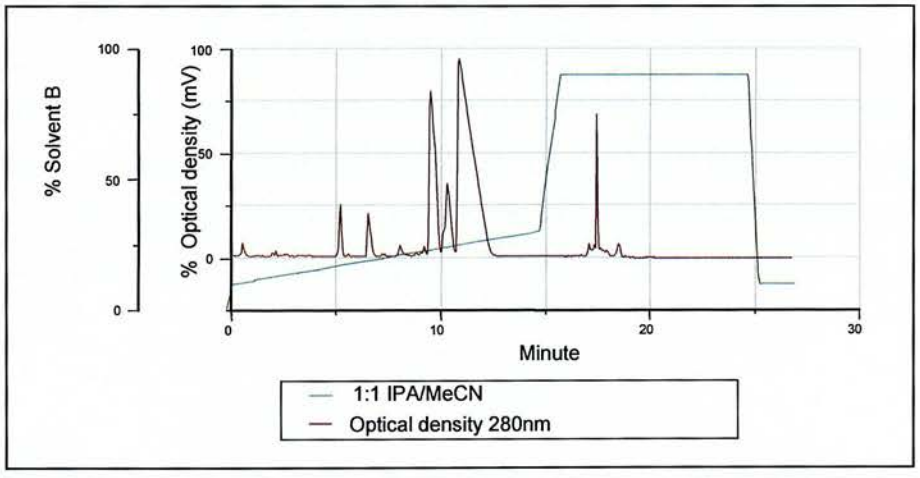


Figure 4.40 Separation on a Phenyl-Hexyl 4.6mm x 150mm with solvent A as 0.1% H_3PO_4 and solvent as B1:1 IPA/ MeCN injecting 2µl sample.

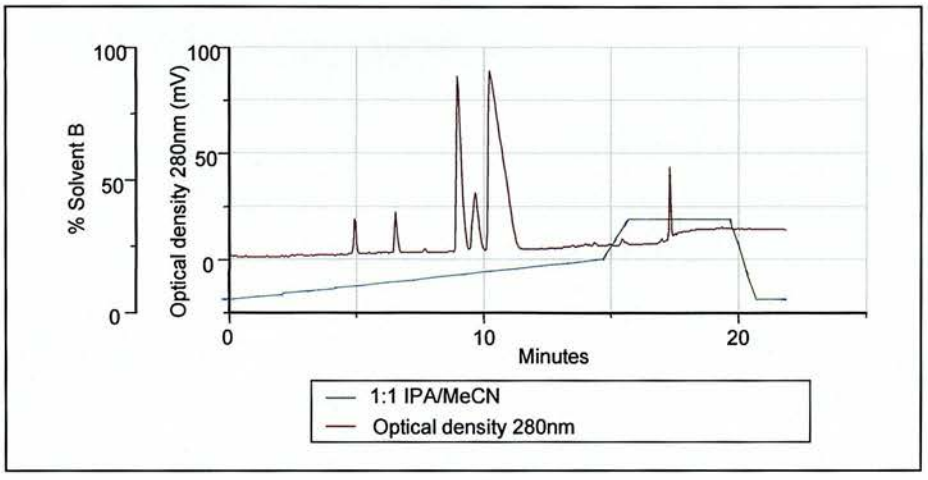


Figure 4.41 Separation on a Phenyl-Hexyl 4.6mm x 150mm with solvent A as 10mM KH_2PO_4 and solvent as B1:1 IPA/ MeCN injecting 1µl sample.

Both runs give a similar elution profile indicating that TFA is not necessary and that other acidic mobile phase modifiers can be used to achieve the same effect. Thus the conditions for improved separation appear to be the use of a phenyl/hexyl stationary phase and an acidic mobile phase containing isopropanol. With this is mind a method was developed to allow the use of mineral acids whilst preventing precipitation of salts in the column. The method uses a solvent gradient similar to above followed by a 5 minute wash with 75% water to remove salts before the high solvent B elution which elutes remaining peaks and regenerates the column. In this way multiple preparative runs could be carried out in succession.

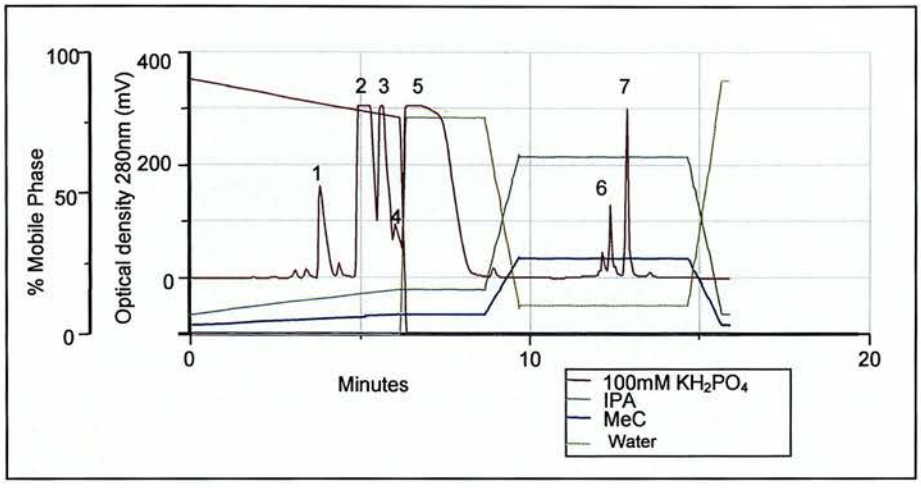


Figure 4.42 Separation on a Phenyl-Hexyl 4.6mm x 150mm column using a 4 part gradient with solvent A as 10mM KH_2PO_4, solvent B as IPA, solvent C MeCN and solvent D H_2O injecting 50µl phase extracted bark extract.

The run was repeated 10 times injecting a total of 0.5ml extract (equivalent to 14.9mg phase extracted *L. amara* alkaloids or 72mg crude *L. amara* bark solids). Each peak was collected into individual tubes and after removing organic solvent in a rotary evaporator was made to ≥1M KCl, extracted into chloroform and back extracted into water to remove traces of KCl before freeze-drying and weighing. Each peak was taken up in 100µl H_2O and assayed in wells. Zones of inhibition were as follows:

Peak number	Zone of inhibition (mm)
1	0
2	0
3	0
4	0
5	16
6	0
7	0

Table 4.16 Active peaks from 10mM KH_2PO_4 phenyl/hexyl column separation.

Active peak 5 consisted of 7.23mg white solid which was identified as lunacridine by ^{1}H NMR (see below). Peak 7 was also collected and treated in the same way providing sufficient material for NMR identification as the related alkaloid lunacrine (see section 4.7 for NMR correlations). Next the same procedure was repeated replacing KH_2PO_4 with HCl, H_2SO_4 H_3PO_4 TFA and CHOOH as the mobile phase modifier and in each case collecting the same characteristically sail shaped peak (peak 5). All apart from acetic acid gave good separation equivalent to the KH_2PO_4 run shown above and this was overcome by taking a slice from the centre of the lunacridine peak.

Finally a run was carried out using a 300mM KCl mobile phase to see what effect high salt concentration would have on the separation when using a standard C-18 column; it was reasoned that since salt promoted the partition of *L. amara* alkaloids into chloroform it might increase the interaction of the analytes with the stationary phase. The mobile phase, which contained no acidic modifiers and was therefore highly inert, gave base line resolution of the bark constituents. The improvement in separation was an unexpected finding since there is no mention in the literature of the use of KCl in combination with a reverse phase stationary phase. It is possible that by somehow increasing the interaction of the quinolines with the stationary phase in analogy to the chloroform phase extraction, the peak tailing effect was overcome. Another possibility is that the high salt concentration acted like a cation exchange mobile phase, overcoming the electrostatic interactions with free silanol groups and so allowing the quinolines to bind in a purely reverse-phase manner.

In an attempt to discern whether lunacridine was the sole anti-*S. aureus* constituent in the bark, 5g fresh (un-dried) *Lunasia* bark was thoroughly extracted with 10ml ethanol, evaporated to dryness and taken up in 1ml 10% ethanol. 25µl were run with the KCl method (Figure 4.43) and each peak collected and assayed in wells along with a control of 25µl of the extract.

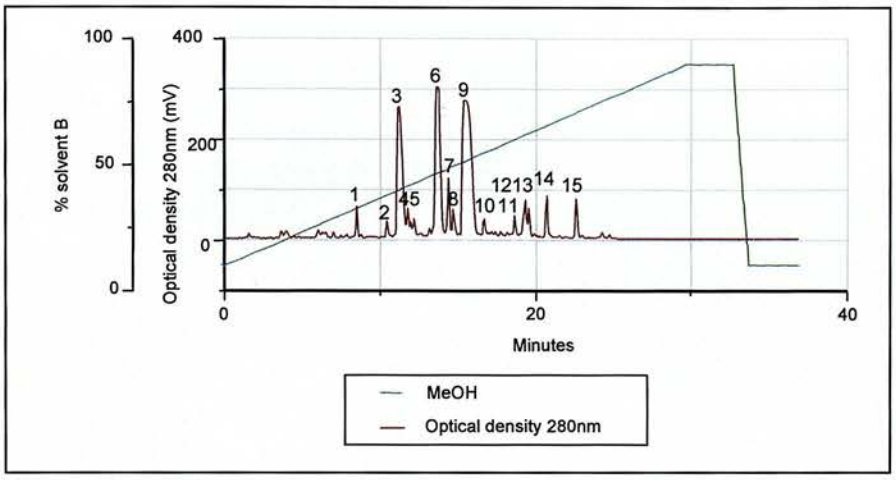


Figure 4.43 Separation on a C-18 5µ 4.6mm x 150mm column with solvent A as 300mM KCl and solvent B MeOH injecting 25µl non-phase extracted *L. amara* bark extract.

Figure 4.44 Each peak (1-15) was collected manually and dried down to 25µl and assayed in wells. A positive control equivalent to the injected sample and a negative control of 1M KCl (higher in KCl than the most concentrated sample) were included for reference.

The plate (Figure 4.44) shows that peak 9 (lunacridine) is responsible for almost all of the activity of the extract with a very small amount of activity surrounding peak 6.

The method was used as above to inject 50µl samples of extract in multiple runs to prepare several mg of the lunacridine peak for NMR. See section 4.7 for proton NMR spectra.

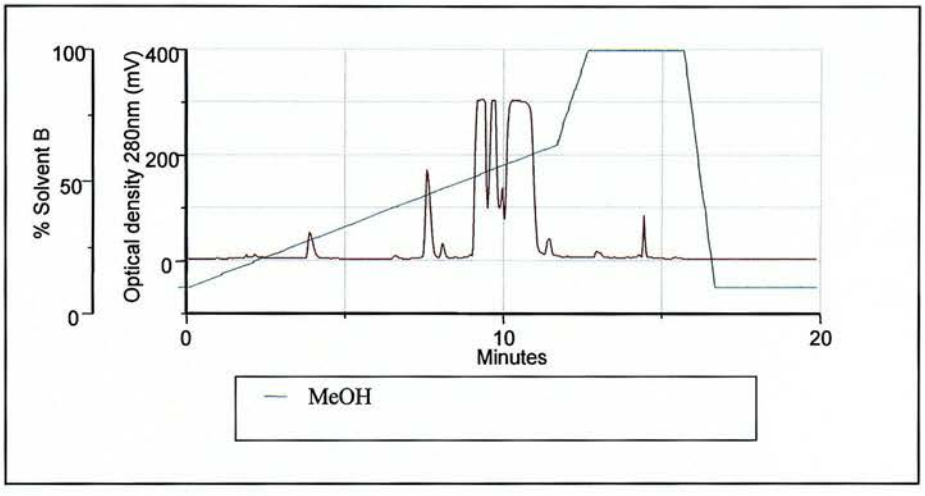


Figure 4.45 Separation on a C-18 5µ 4.6mm x 150mm column with solvent A as 300mM KCl and solvent B methanol, injecting 50µl sample.

4.7 Identification of samples by NMR

Each of the samples were subjected to a proton NMR spectrum to confirm that the sample was in fact lunacridine and to determine if any chemical modification such as trifluoroacetylation and occurred. Shown below are the proton spectra for the samples. For the sample prepared with TFA, trifluoroacetylation of the side chain hydroxyl has occurred and so the sample is the same as the "TFA derivative" isolated previously. Where TFA was replaced with acetic acid lunacridine appears as the corresponding

formyl derivative. Samples prepared with phosphate containing mobile phases and potassium chloride, yielded lunacridine in its native state.

Site	δ_H	proton	J_{HH} (Hz) multiplicity	Notes
5	7.82	(dd)	8.2, 0.8	
6	7.57	('dd')	8.2, 8.0	not fully resolved; appears as t
7	7.44	(d)	8.0, 0.8	small J not resolved
1'a	3.99	(dd)	15.8, 9.0	
1'b	3.65	(dd)	15.8, 7.6	
2'	5.15	('ddd')	9.0, 7.6, 6.7	not fully resolved appears q
3'	2.18	(oc)	6.7	outer lines very low intensities
4'	1.10	(d)	6.7	
5'	1.06	(d)	6.8	
N-Me	4.22*	(s)		
4-O-Me	4.42*	(s)		
8-O-Me	4.01*	(s)		

* = assigned by analogy with spectrum of trifluoroacetyl derivative and so may be interchanged

Table 4.17 ^{1}H NMR Spectral Data for native (un-derivatised) lunacridine prepared with 10mM HCl mobile phase.

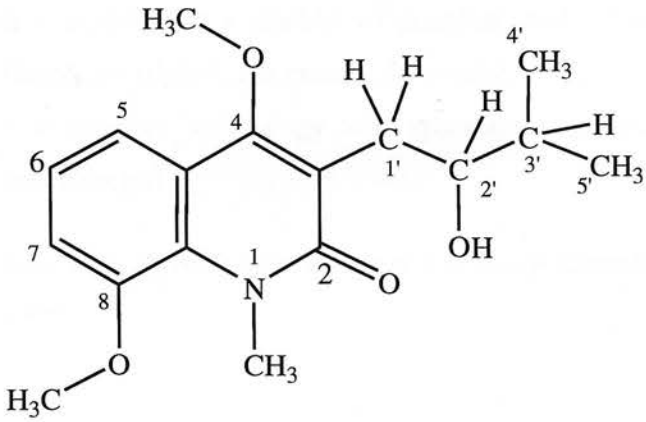


Figure 4.46 Native (un-derivatised) lunacridine as prepared with phosphate and potassium chloride based mobile phases.

Site	δ_H	proton	J_{HH} (Hz) multiplicity	Notes
Formate	8.46	(s)		
5	7.83	(d)	8.3	
6	7.57	(dd)	8.3, 8.0	
7	7.48	(d)	8.0	
1'a	3.99	(dd)	15.9, 9.0	
1'b	3.65	(dd)	15.9, 7.5	
2'	5.15	(ddd)	9.0, 7.5, 6.7	not fully resolved appear as q
3'	2.17	(oc)	6.7	outer lines very low intensities
4'	1.10	(d)	6.7	
5'	1.04	(d)	6.7	
N-Me	4.23*	(s)		
4-O-Me	4.42*	(s)		
8-O-Me	4.00*	(s)		

d = doublet, dd = doublet of doublets, ddd = doublet of doublets of doublets, dt = doublet of triplets, q = quartet, oc = octet, s = singlet, t = triplet

* = assigned by analogy with spectrum of trifluoroacetyl derivative and so may be interchanged

Table 4.18 ^{1}H NMR Spectral Data of 2'-*O*-Formyl-lunacridine prepared using 0.1% acetic acid mobile phase.

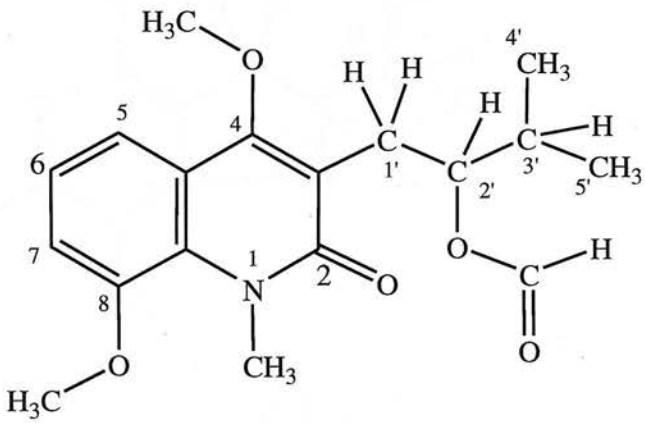


Figure 4.47 2'-*O*-Formyl-lunacridine prepared using 0.1% acetic acid mobile phase.

Site	δ_H	proton	J_{HH} (Hz) multiplicity
5	7.8	(d)	8.2
6	7.4	(t)	7.3, 8.2
7	7.3	(d)	7.3
2´	5.0	(dt)	9.3, 2 x 6.8
1´a	3.3	(dd)	14.9, 9.3

1'b	3.0	(dd)	14.9, 6.8
3'	2.1	(oc)	6.7
4'	1.0	(d)	6.8
5'	1.0	(d)	6.9

Table 4.19 ^{1}H NMR Spectral Data of Lunacrine prepared with KH_2PO_4 mobile phase

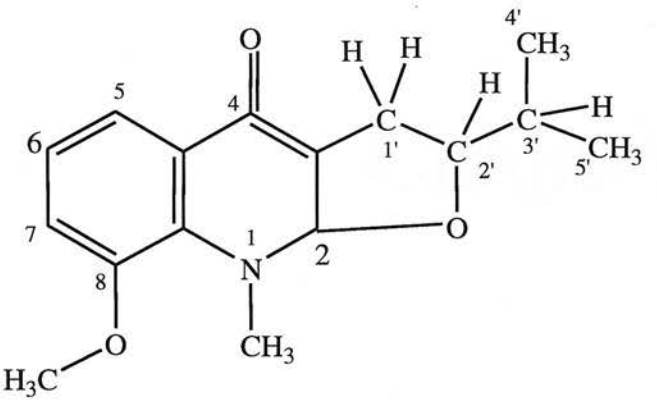


Figure 4.48 Structure of lunacrine prepared with KH_2PO_4 mobile phase.

It was noticed that samples of native lunacridine lost their antibacterial activity after several months even when stored at -20°C. ^{1}H spectra of both old and fresh lunacridine prepared under identical conditions were recorded and these showed the presence of increasing quantities of an impurity corresponding broadly with lunacrine (data not shown) suggesting cyclisation of lunacridine occurs even at low temperatures. Loss of activity was not observed with the formate and trifluoroacetyl derivatives (presumably since capping of the side chain hydroxyl prevented cyclisation) and so for subsequent experiments the 2'-*O*-Trifluoroacetyl derivative here to referred to TFA lunacridine was used.

Chapter 5

BIOLOGICAL ACTIVITIES

5.1 Antibacterial activity

MIC experiments were carried out with *S. aureus* NCTC 6571 and *E. coli* ATCC 10536 to gain a more quantitative measure of the antibacterial activity of the bark extracts and the 2'-*O*-Trifluoroacetyl-lunacridine compound (Table 5.1). For comparison, certain related compounds (nalidixic acid, etoposide and chloroquine) were included. The agar dilution technique described by (Andrews, 2001) was used for the bark extracts and structurally related compounds but it was necessary to use the microdilution method (Andrews, 2001) for the pure compound since only small quantities were available.

Aqueous and ethanolic extracts were prepared by thorough extraction of 5g finely powdered bark with 100ml ethanol or water, filtering through Whatman No. 1 paper followed by rotary evaporation or freeze-drying. This yielded 0.5515g and 0.7247g solids respectively which were taken up in ethanol and water to make 10mg/ml solutions before filtering through a 0.2μ syringe tip (Sartorius) to sterilise.

Drug or extract	MIC μg/ml *S. aureus* NCTC 6571	MIC μg/ml *E. coli* ATCC 10536
Crude ethanolic extract	128	>256
Crude aqueous extract (freeze-dried)	256	>256
Etoposide	32	>256
Chloroquine diphosphate	>256	>256
TFA lunacridine	64 (by microplate dilution)	>256
Nalidixic acid sodium salt	4	64

Table 5.1. MICs of aqueous and ethanolic *L. amara* bark extracts along with pure 2'-*O*-Trifluoroacetyl-lunacridine and certain related compounds.

The MIC data show lunacridine to be inactive against *E. coli* and only weakly active against *S. aureus* with an MIC of 64μg/ml. Such activity is well below that of clinically

used antibiotics so it is unlikely lunacridine would show much potential in this area. It was instead decided to look at lunacridine's mode of action and determine what other activities it possessed.

Lunacridine bears structural similarities to the antibacterial quinolone class of compounds epitomised by the founding member of the class nalidixic acid. This similarity in structure led to the suspicion that lunacridine, like its quinolone relatives is an inhibitor of bacterial topoisomerase(s). To test this theory lunacridine was assayed against strains of the gram positive bacterium *Streptococcus pneumoniae* differing in their sensitivity to penicillin, erythromycin and the quinolone antibiotic ciprofloxacin (Table 5.2). The assay was carried out by Professor S. Amyes (Department of Medical Microbiology, University of Edinburgh) who had obtained stocks of clinically acquired strains with varying susceptibilities to the antibiotics. Disks containing 200μg lunacridine were screened against the strains using the Kirby/ Bauer filter paper disk diffusion assay. *S. pneumoniae* strains were cultured according to NCCLS guidelines at 37°C, 5% CO_2, on Mueller-Hinton agar supplemented with 5% defibrinated horse blood.

Compound/Strain	25306	3018	SZ 27033	ATCC49619
Lunacridine ZOI	14	9	15	16
Penicillin MIC	8	0.015	1	sensitive
Erythromycin MIC	512	256	64	sensitive
Ciprofloxacin MIC	1	4	2	sensitive

Table 5.2 TFA lunacridine zones of inhibition (mm) on strains of *S. pneumoniae* with varying sensitivities to penicillin, erythromycin and ciprofloxacin. MICs expressed as μg/ml.

The sensitive reference strain ATCC49619 gave the largest zone of inhibition whereas strain 3018 which has a raised MIC towards both erythromycin and ciprofloxacin gave a considerable decrease in the lunacridine zone size from 16mm down to just 9mm showing that lunacridine's antibacterial potency is reduced with quinolone resistant strains. Strain 25306 which has a raised MIC of 8μg/ml for penicillin and a high MIC of

256μg/ml for erythromycin showed only a small decrease in the lunacridine zone size from 16mm for the sensitive strain down to 14mm. Strain SZ 27033 had a raised MIC of 64μg/ml for erythromycin and but much smaller increases in MIC towards penicillin and ciprofloxacin but did not produce the same dramatic drop in zone size seen with 3018 thereby removing the possibility that macrolide resistance was responsible for the 9mm zone size seen with quinolone resistant strain 3018. With strain ATCC 49619 numerous colonies were seen around the rim of the zone of inhibition indicating the presence of lunacridine resistant mutants.

5.2 DNA intercalation

The planar-cationic aromatic structure of lunacridine lead to the suggestion that it might act as a DNA intercalator since such compounds are known for their ability to slot between base pairs and complex with DNA (Lerman, 1961). The phenomenon was first investigated with the acridine dyes by L.S Lerman in 1961 who postulated a mechanism in which untwisting of the double helix provides sufficient space between the layers of bases to allow intercalation. To test the idea, a DNA intercalation assay was set up using the method described by Cain et al 1978 (Cain et al., 1978). The assay is based on a ligand displacement type approach which makes use of the increased fluorescence of complexed ethidium Br/DNA over free ethidium Br. In the assay the fluorescence of intercalated ethidium Br DNA is decreased when displaced by a competing intercalator. Care was taken to determine that compounds did not absorb at either the excitation or emission wavelengths (546 and 595nm) at the highest concentration reached in the assay.

To begin with, the known intercalating agents amsacrine and chloroquine were used and both agents produced the anticipated decrease in fluorescence giving IC_{50} figures in broad agreement with those obtained by Cain et al (Cain et al., 1978) although the figure for amsacrine was slightly lower than expected (Figure 5.1). As predicted 2'-*O*-trifluoroacetyl-lunacridine also caused a reduction in ethidium DNA fluorescence

although this occurred at a markedly higher concentration than with amsacrine or chloroquine. The experiment was also carried out using the prototype quinolone nalidixic acid. Whilst quinolones such as nalidixic acid are known to bind to DNA (Sandstrom et al., 2003) this does not occur through an intercalative type mechanism and as expected high concentrations (0.7 mM) failed to illicit any decrease in fluorescence. The effect was investigated further the by using DNA pre-incubated in 0.5mM nalidixic acid and adding increasing concentrations of chloroquine. The resulting graph is near identical to that obtained with chloroquine alone, indicating the nalidixic acid binding site is located distal to the chloroquine site of intercalation.

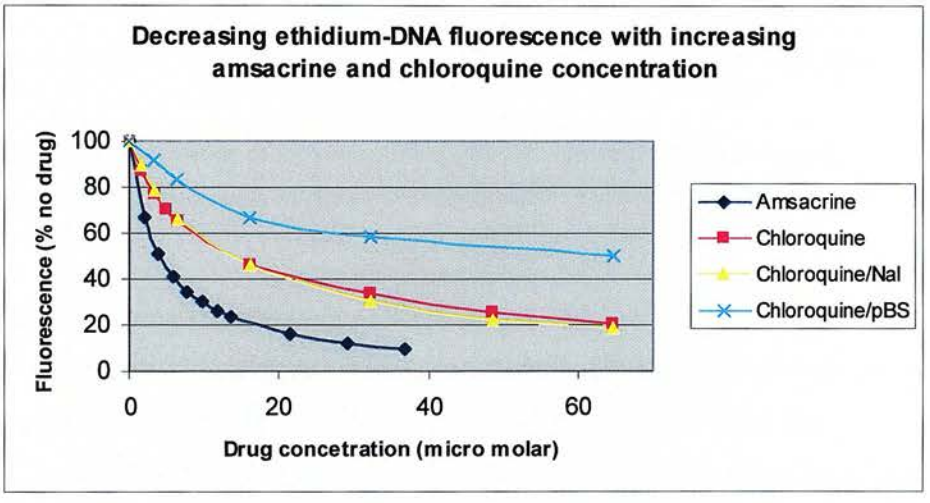


Figure 5.1 Intercalation of chloroquine, chloroquine and 0.5mM nalidixic acid and amsacrine with calf thymus and pBS plasmid DNA.

Lunacridine intercalation data was also collected replacing calf thymus DNA with pBS plasmid (Figure 5.2). As expected intercalation occurred less easily with the plasmid, its closed circular structure acting to resist unwinding of the helix which is necessary for intercalation of successive molecules; the same effect was observed with chloroquine and amsacrine.

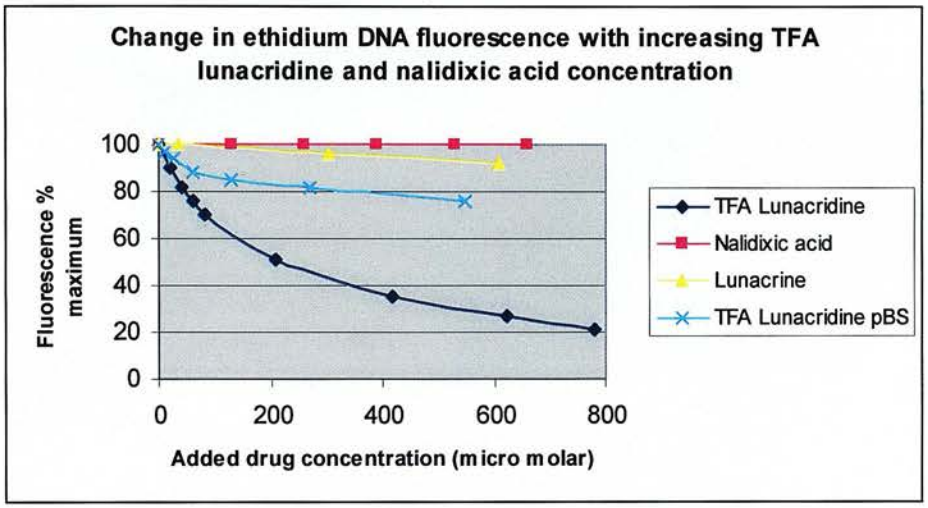


Figure 5.2 Intercalation of TFA lunacridine with calf thymus DNA and pBS plasmid. Minimal and no intercalation are seen with related compounds lunacrine and nalidixic acid respectively.

Samples prepared with each of the different HPLC mobile phase additives were assayed to determine their relative activity (Figure 5.3). All of the samples prepared with salts (KH_2PO_4, HCl, H_2SO_4 and KCl) are native lunacridine whereas those prepared with TFA and CH_3COOH were modified at the side chain hydroxyl to form ester derivatives. The modified samples proved to be the most efficient intercalators whereas the samples prepared with mineral acids showed varying degrees of intercalation despite identification by NMR as highly pure lunacridine. This further supported the use of the 2'-*O*-Trifluoroacetyl derivative rather than the native compound which appeared to be unstable.

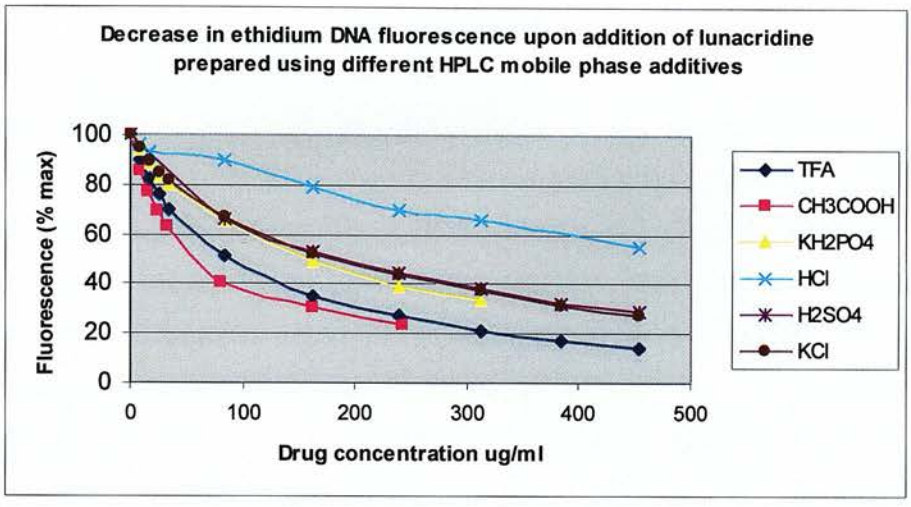


Figure 5.3 Comparison of intercalative ability of various lunacridine samples prepared with different HPLC mobile phase additives. The ester derivatives which lack a free hydroxyl group are the most active.

Compound	Sample DNA	IC_{50} intercalation (μM)
Amsacrine	Calf thymus	4
Chloroquine	Calf thymus	14
Chloroquine	pBS plasmid	64.6
Chloroquine	Calf thymus pre-treated with 0.5mM Nalidixic acid	13.5
TFA Lunacridine	Calf thymus	220
TFA Lunacridine	pBS plasmid	822.8
Lunacridine formate ester	Calf thymus	160
Lunacrine	Calf thymus	>1144
Nalidixic acid	Calf thymus	>660

Table 5.3 DNA intercalation IC_{50} of each compound with calf thymus linear DNA or pBS plasmid (derived from previous graphs).

5.3 Topoisomerase inhibition

A logical progression from the identification of lunacridine as a DNA intercalator was to test the compound in a human topoisomerase II inhibition assay since there is a relationship between DNA intercalation and inhibition of the decatenating enzyme (Brana et al., 2001). Moreover, a planar cationic ring system along with an "interfering side chain" are two important characteristics of the topoisomerase poison group of inhibitors (Capranico and Binaschi, 1998). The topoisomerases are a family of enzymes dedicated to controlling the degree of supercoiling present in the DNA of both eukaryotic and prokaryotic cells (Sinha, 1995). Mammalian topoisomerase II belongs to the ATP dependent type II group of enzymes and is able to perform a range of functions including the decatenation of linked concatemers and the removal of both negative and positive supercoils from DNA. Mammalian topoisomerase II has been shown to be vital for a variety of cellular processes including replication, transcription, recombination and chromosome segregation (Bromberg and Osheroff, 2001).

The possibility of an interaction with topoisomerase was supported by the experiments with *S. pneumoniae* that showed antibacterial activity was reduced in bacteria that were resistant to ciprofloxacin which is known to target type II bacterial topoisomerases (Anderson et al., 1999). Although bacterial type II enzymes are distinct from their mammalian counterparts certain drugs such as etoposide and novobiocin are inhibitors of both enzymes. To test the theory a topoisomerase II assay kit was purchased from TopoGEN Inc, Columbus, Ohio, USA along with recombinant human topoisomerase II alpha (p170). The assay is based on the ability of the enzyme to decatenate kinetoplast DNA (kDNA) which is composed of numerous interlinking concatemers of closed circular DNA. The kDNA is too large to migrate on an agarose gel so forms an intense band adjacent to the leading edge of the well it is loaded into, but in the presence of enzyme and ATP, the kDNA substrate is de-concatemerized into monomers that are small enough to migrate down the gel. To test the kit, reactions were run for 1 hour at 37°C in the presence of varying concentrations of amsacrine which is a known

topoisomerase II poison. The gel shows no inhibition at 0.5μM demonstrated by the presence of a decatenation product band but complete inhibition at 1μM where only the immobile kDNA band is visible (Figure 5.4). This is in broad agreement with figures reported for amsacrine cleavable complex formation (Nelson, 1983; Patel et al., 2000).

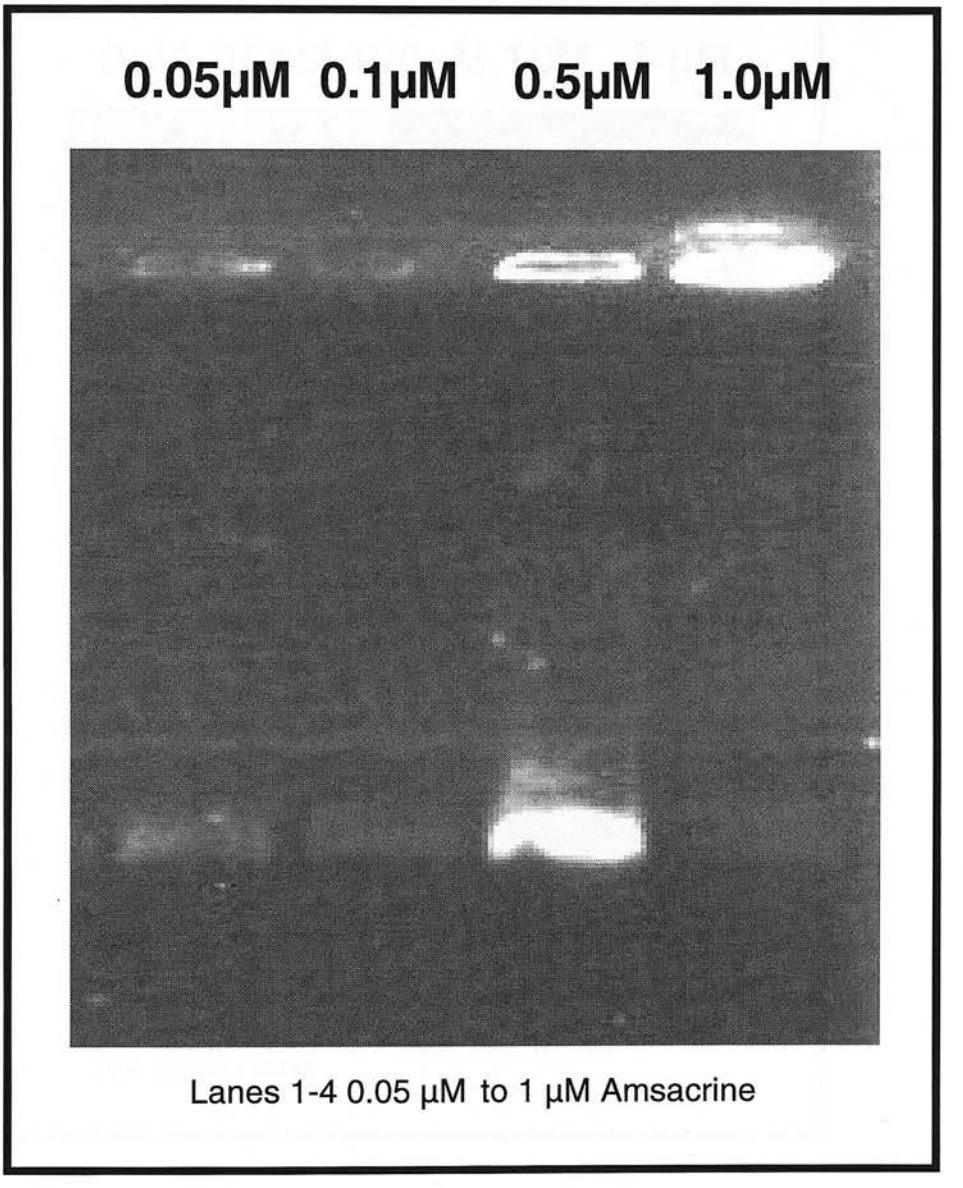


Figure 5.4 Human topoisomerase II alpha incubated with amsacrine between 0.05μM and 1μM. Complete inhibition of decatenation occurs between 0.5 and 1μM.

The experiment was then repeated with TFA lunacridine at 10nm to 5μM to look for similar signs of inhibition to those seen with amsacrine. As can be seen in the gel below there is a clear cut off between 1 and 5μM with pronounced inhibition of decatenation visable at 5μM.

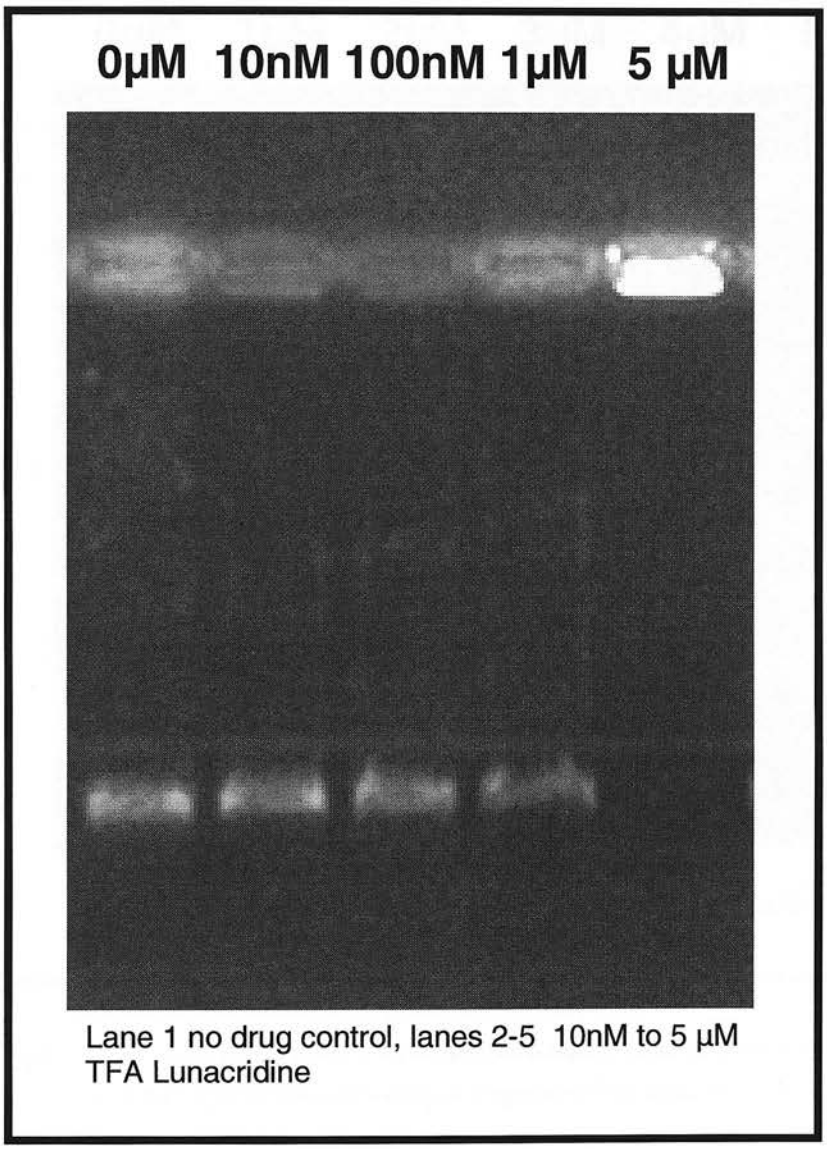


Figure 5.5 Human topoisomerase II alpha incubated with TFA lunacridine between 10nM and 5μM with inhibition occurring between 1μM and 5μM.

The assay was repeated with a narrower range of concentrations. As can be seen from the gel there is a sudden drop in topoisomerase II activity between 4μM and 5μM TFA lunacridine (Figure 5.6). The experiment was repeated twice each giving the same result.

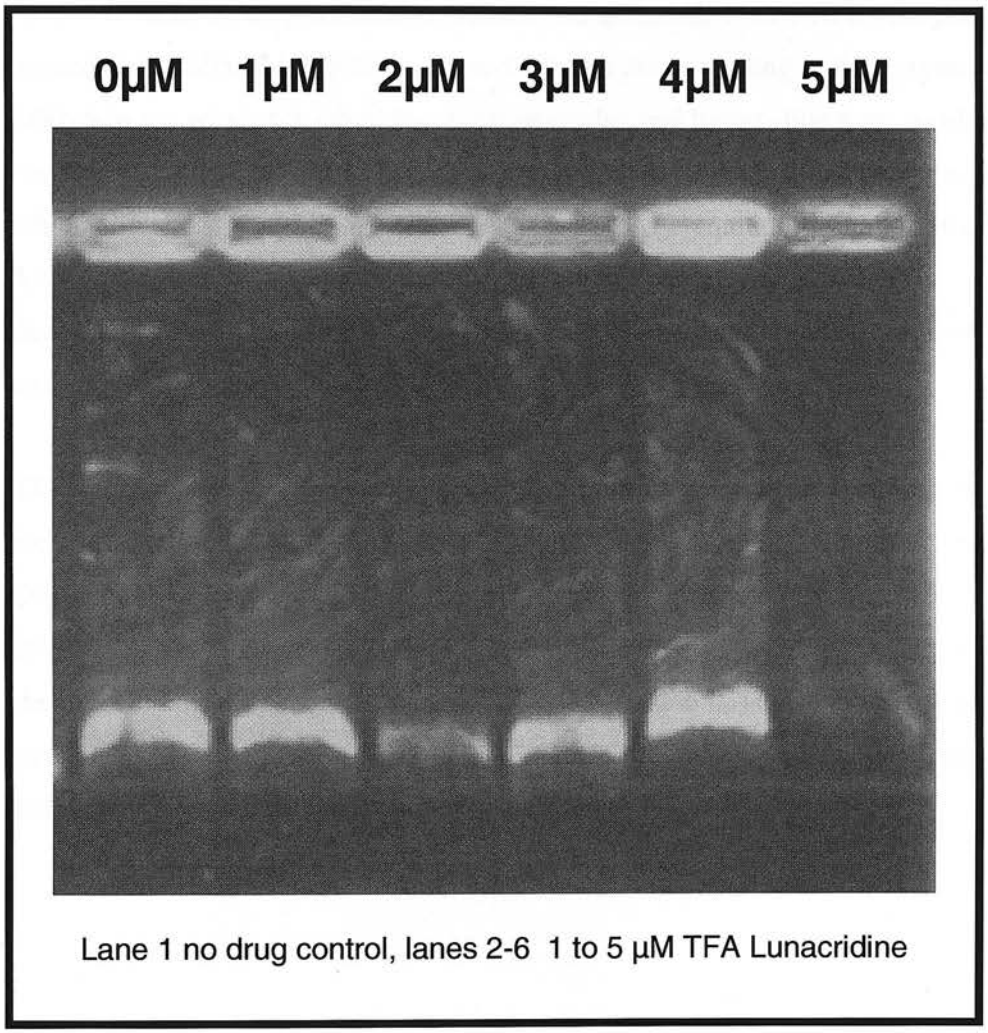


Figure 5.6 Human topoisomerase II alpha incubated with TFA lunacridine between 1 and 5μM with inhibition occurring between 4μM and 5μM. Experiment was repeated twice.

Inhibitors of mammalian topoisomerase II may act as catalytic inhibitors by targeting the enzyme itself (a mode of action typified by the coumarin class of drugs) or may act as a topoisomerase poison and trap the enzyme on its DNA substrate e.g. amsacrine and numerous other DNA intercalators (Jensen and Sehested, 1997). To investigate whether lunacridine mediated inhibition occurred through direct binding to the enzyme or if DNA was necessary for inhibition, both amsacrine and lunacridine were incubated with enzyme alone for 10 minutes before addition of kDNA substrate and reaction buffer which diluted the drugs to below the levels previously required for inhibition (Figure 5.7) . The rest of the assay was then carried out as before. Inhibition of deconcatermerisation did not occur despite both drugs being initially present at twice the concentration previously required for inhibition.

The result shows that inhibition does not occur through direct covalent binding to the enzyme, a more likely mode of action being topoisomerase poisoning in which enzyme, DNA and drug are trapped together in a tripartite complex. Although certain DNA intercalators such as aclarubicin are able to inhibit topoisomerase II by simply hindering the interaction of enzyme and substrate (Larsen et al., 2003) this would appear unlikely in the case of lunacridine since complete enzyme inhibition occurs at concentrations below which significant DNA binding takes place.

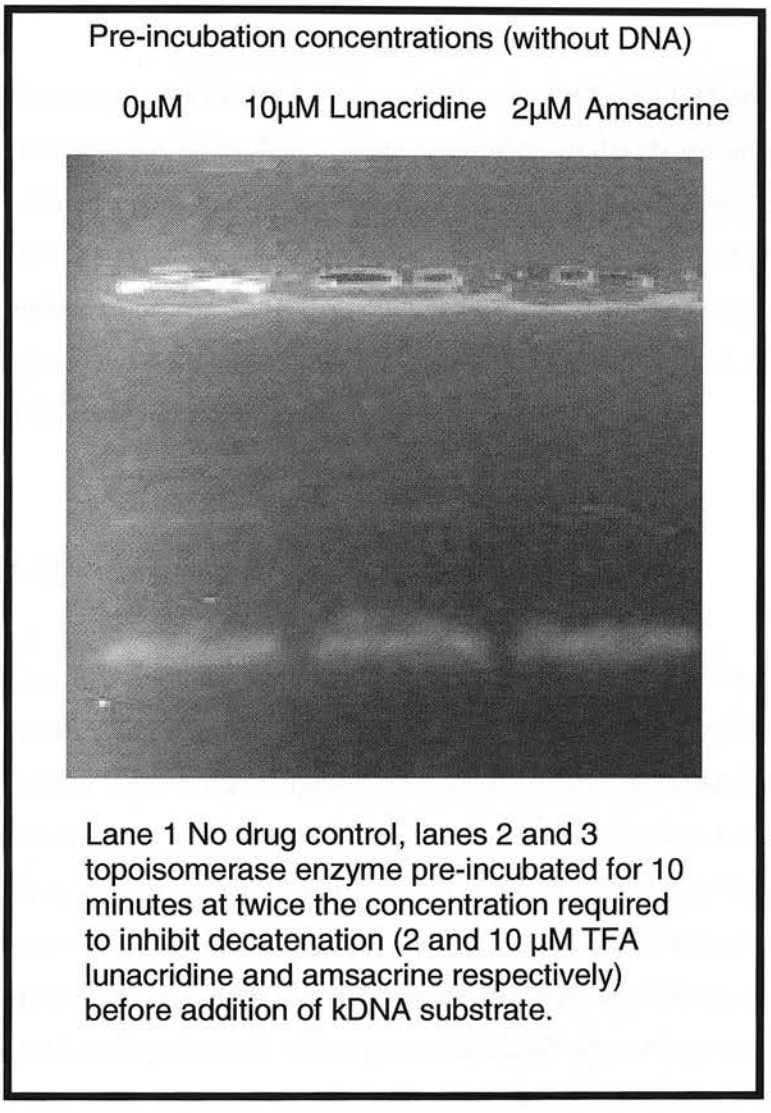


Figure 5.7 Decatenation products after pre-incubation of enzyme with 2μM and 10μM amsacrine and TFA lunacridine respectively. kDNA and reaction buffer were then added to dilute the drugs below the 1μM and 5μM needed for inhibition and after 1 hours incubation at 37°C the reaction products were run on a gel as before. Decatenation was able to take place despite initial inhibitor concentration being twice what was required for inhibition in the presence of kDNA substrate.

5.4 Potential anti-viral activities

Several lines of evidence suggested that lunacridine might posses some degree of anti-viral activity, either against certain members of the *Herpesviridae* which depend on cellular topoisomerase II for replication (Bailly, 2000) or against retroviruses such as HIV-1 since quinolones and certain DNA intercalators have been shown to inhibit the reverse transcriptase (RT) (Tomita and Kuwata, 1976). Experiments were therefore carried out with herpes simplex virus 2 (HSV-2) and HIV-1 reverse transcriptase to see if this was indeed the case.

5.4.1 Reverse transcriptase inhibition activity

Certain quinolones such as nalidixic acid as well as some intercalators (Aoyama, 1991), (Tomita and Kuwata, 1976) have been reported to act as inhibitors of the virally encoded enzyme reverse transcriptase (RT). To establish if lunacridine possessed such activity a RT assay kit was purchased from Roche Diagnostics Ltd, Lewes UK. The assay is based on an ELISA format in which biotin and digoxigenin labelled nucleotides are incorporated into DNA by RT working on a poly A x oligo $(dT)_{15}$ hybrid template primer. The resulting DNA is bound to the surface of streptavidin coated wells. Bound DNA is then detected with a peroxidase antibody conjugate (anti-DIG-POD) which binds to the digoxigenin labelled nucleotides and produces a coloured reaction product upon addition of 2,2¢ -azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt (ABTS) substrate. The kit was tested with chloroquine, nalidixic acid and TFA lunacridine up to 20 mM (Figure 5.8) incubating each drug for at 37°C for one hour in the presence of HIV-RT enzyme, template primer and a reaction buffer provided with the kit (for more details see section 2.13). The reaction contents were then transferred to the specially provided streptavidin-coated microplates and after repeated cycles of

washing, antibody was added and the plates read colourmetrically. RT activity was plotted as % no-drug control.

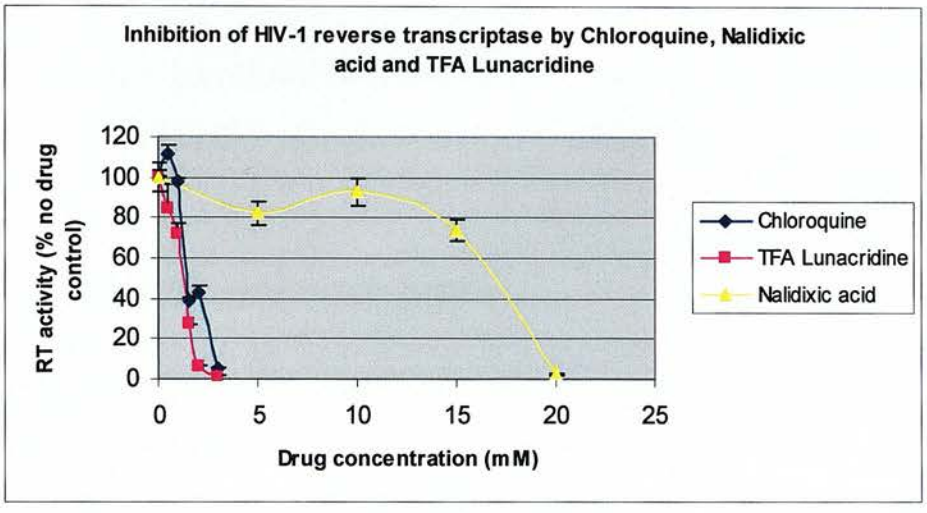


Figure 5.8 Inhibition of HIV-1 RT by TFA lunacridine, chloroquine diphosphate and nalidixic acid. Results are expressed as a percentage of no-drug control carried out independently for each drug and are an average of 4 data points for each drug concentration.

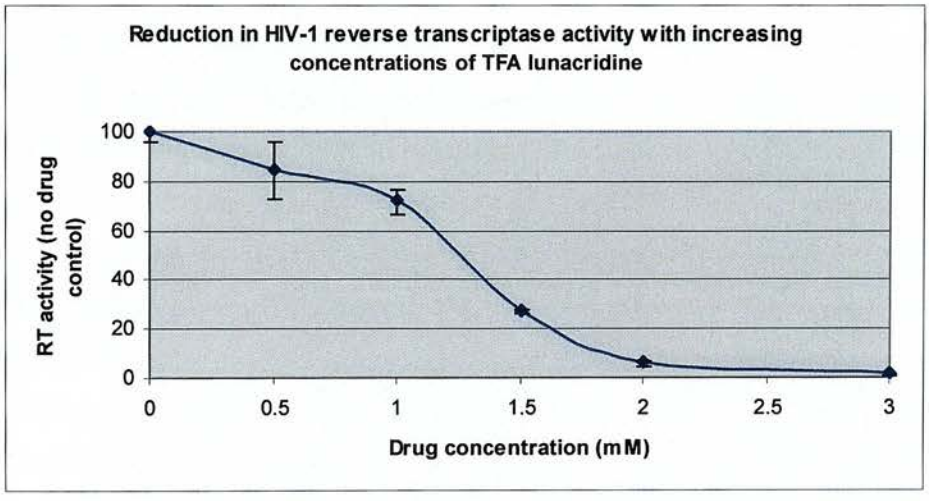


Figure 5.9 Inhibition of HIV-1 RT by TFA Lunacridine. Results are expressed as a percentage of no-drug control carried out independently for each drug. Results are an average of 4 data points for each drug concentration.

The results show a clear inhibition of HIV-RT enzyme activity for all of the drugs, lunacridine being the most potent of the three (IC_{50} 1.25mM). The effect is clearly not simply due to intercalation with the template primer since chloroquine which is a far more efficient intercalator is less active than lunacridine.

5.4.2. Anti-herpes simplex virus activity

HSV-1 and HSV-2 replication is dependent on cellular topoisomerase II; the HSV-1 genome in particular contains numerous topoisomerase II recognitions sites which are acted on late in the infective cycle (Erbert et al., 1990;1994). There is therefore some interest in using topoisomerase II inhibitors such as those developed as cancer drugs as inhibitors of the viruses (Bailly, 2000). A stock solution of TFA lunacridine was sent to Dr M. Ogilvie at the Specialist Virology Centre, Edinburgh Royal Infirmary to asses its potential as an anti-HSV-1 agent.

TFA lunacridine was evaluated using the virus yield reduction assay, which was chosen instead of the more commonly used plaque reduction assay as it is a more sensitive technique using a multiplicity of infection of 0.01 plaque forming units (PFU) per cell compared with a multiplicity of infection of 1 or 2 PFU for the plaque reduction assay. This allows the detection of antiviral activity more rapidly and over a wider range of drug concentrations. Plates treated with acyclovir showed a 100% reduction in virus yield as expected but TFA lunacridine showed no reduction relative to uninfected controls even at 15.63μM the highest concentration used (data not shown). The drug could not be used above this concentration due to cytotoxic effects so the study was not taken any further.

5.5 Cytotoxicity and induction of apoptosis in human cell lines

The low micro molar concentration required for inhibition of human topoisomerase II by TFA lunacridine pointed towards a degree of cytotoxicity in human cell lines since topoisomerase II inhibitors are widely employed as cytotoxic agents in cancer chemotherapy (Sinha, 1995). Topoisomerase II poisons are known to cause double strand breaks in cellular DNA (Jensen and Sehested, 1997) and if such breaks are not repaired they may trigger the apoptotic pathways that lead to programmed cell death (Kaina, 2003; Sordet et al., 2003). This activity is exploited by cancer drugs such as amsacrine and etoposide which are able to produce selective killing of tumour cells (Dolega, 1998). It is thought that such selective cytotoxicity is possible because cancer cells divide more rapidly than healthy cells and so undergo replication more frequently (Marchini et al., 2004); the increased topoisomerase II activity resulting in increased DNA damage (De Beer et al., 2001; Holden, 2001; Lock and Ross, 1987). Since topoisomerase II is an important cancer drug target, (Sinha, 1995) cytotoxicity assays were carried out with different cell lines to determine if lunacridine showed any degree of selective cytotoxicity towards carcinoma cells.

Three cell lines were selected namely ATCC MRC-5, NCI H226 and HELA. MRC-5 are non-carcinoma lung cell fibroblasts derived from foetal lung tissue and reach senescence in 42 to 48 doublings (Jacobs et al., 1970) whilst H226 are an immortal non-small cell lung carcinoma line. HELA are epithelial cervical carcinoma cells (Scherer and Hoogasian, 1954) and the fastest growing of the three cell lines. The cells were treated with lunacridine and the known topoisomerase II poison etoposide at concentrations up to 100μM and left for time varying time intervals ranging between 4 and 72 hours. After exposure to the drugs, cell viability was measured using the Cell Titer 96 Aqueous One solution cell proliferation reagent (Promega) which contains the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS[(a)]] and an electron coupling reagent (phenazine ethosulfate;

PES). Reduction of the reagent as a result of cellular metabolic activity results in the production of a coloured formazan product whose absorption at 490nm is directly proportional to the number of living cells. The cells were grown in optical 384 well tissue culture treated microplates which were read colourmetrically at 490nm in a plate reader. Cell viability was expressed as % of no drug controls after subtracting background absorbance. The following graphs depict the decreasing cell viability of each of the three cell lines with increasing exposure to the two drugs. Cell viability is expressed as a percentage of no drug controls and each drug concentration was repeated 8 times. Only positive error bars (standard deviation) are shown for clarity.

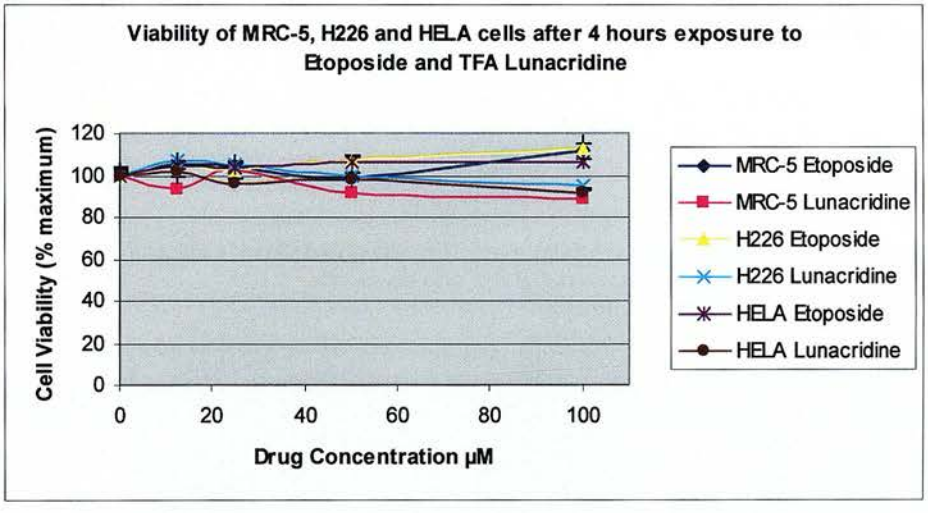


Figure 5.10 Viability of MRC-5, H226 and HELA cells exposed to etoposide and TFA lunacridine for 4 hours. Cells were trypsinised and plated into transparent 384 well plates, allowed to attach overnight before adding serially diluted drugs. The plate was then incubated for 4 hours before addition of Cell Titer reagent and incubated for a further 2 hours before reading colourimetrically at 490nm.

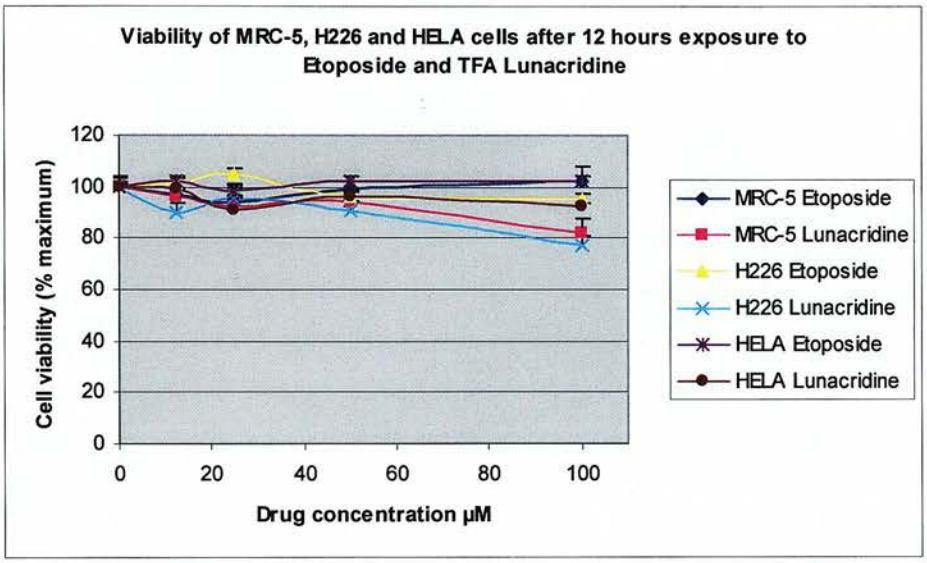


Figure 5.11 Viability of MRC-5, H226 and HELA cells exposed to etoposide and TFA Lunacridine for 12 hours.

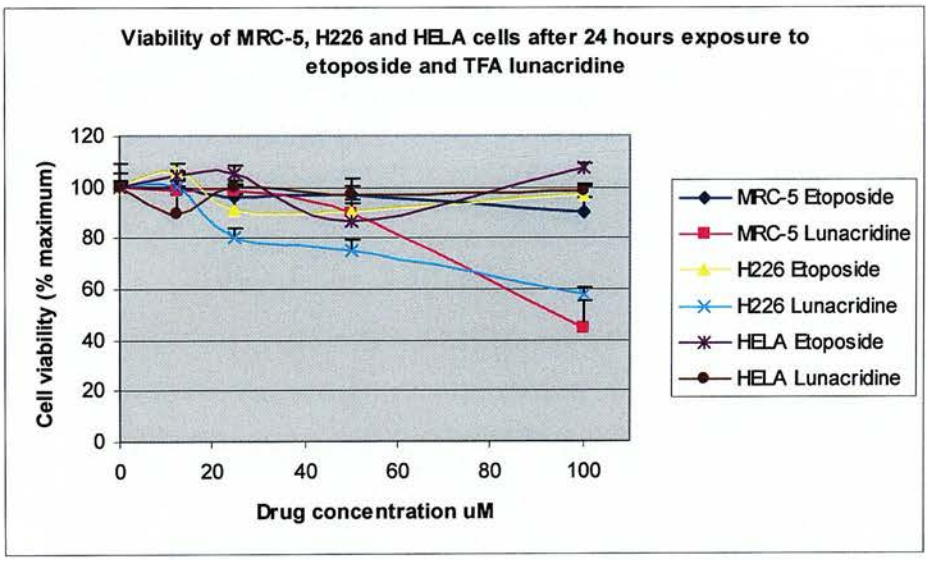


Figure 5.12 Viability of MRC-5, H226 and HELA cells exposed to etoposide and TFA Lunacridine for 24 hours.

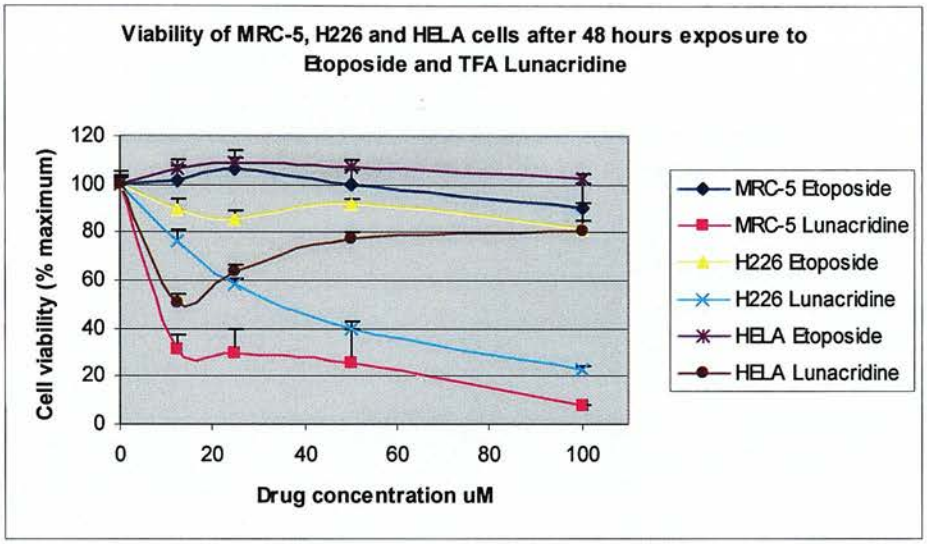


Figure 5.13 Viability of MRC-5, H226 and HELA cells exposed to etoposide and TFA Lunacridine for 48 hours.

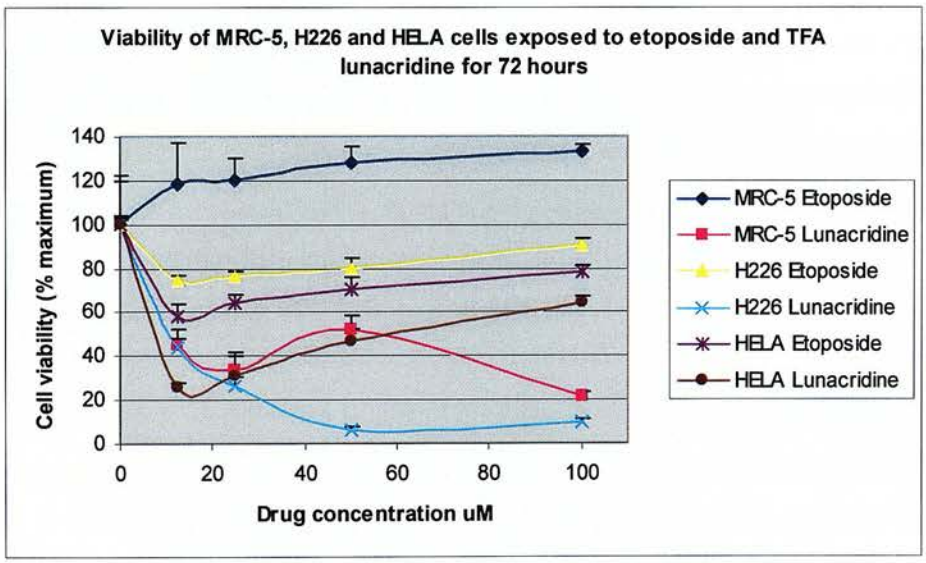


Figure 5.14 Viability of MRC-5, H226 and HELA cells exposed to etoposide and TFA Lunacridine for 72 hours.

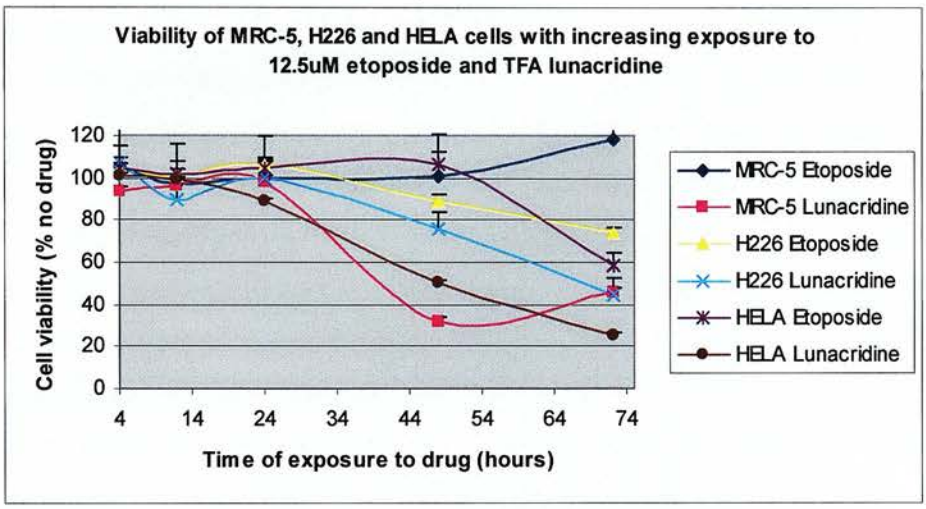


Figure 5.15 Viability of MRC-5, H226 and HELA cells exposed to 12.5μM etoposide and TFA Lunacridine between 4 and 72 hours (data derived from previous graphs).

The results clearly show a downwards progression in cell viability with increasing drug concentration for lunacridine but unfortunately there is little difference between the carcinoma cell lines (H226 and HELA) and non-carcinoma cell line MRC-5. This is not the case with etoposide which as expected exhibits selective inhibition of the carcinoma cell lines showing little inhibition of MRC-5 cells even after 72 hours exposure to 100μM concentration of the drug (Figure 5.14).

Despite this it was decided to repeat the experiment replacing the CellTiter reagent for a caspase detecting reagent to ascertain if the reduced cell viability was due to a cell necrosis type mechanism or, as is the case with other topoisomerase II poisons an apoptotic cell death mechanism (Figure 5.16) (Kiechle and Zhang, 2002).

Apoptosis is the programmed cell death of damaged cells and is distinct from cellular necrosis in that it represents a specific and programmed biochemical process which is dependent on key effectors in the apoptotic pathway and therefore takes time to complete; necrosis on the other hand occurs rapidly after the initial insult (Philchenkov, 2004). Caspases (cysteinyl aspartate-specific proteases) are key effector molecules in the apoptotic pathway that become activated in response to DNA damage such as topoisomerase II poison induced double strand breaks (Philchenkov, 2004). Caspases can be divided into two groups based around their role in the complex apoptotic pathways either as initiators (caspases 2, 8, 9 and 10) or effector caspases (3, 6 and 7) (Philchenkov, 2004). Effector caspases are activated by initiator caspases and once active are able to cleave a wide range of substrates which include crucial structural and regulatory proteins thereby "disassembling" the cell (Philchenkov, 2004).

The Apo-1 reagent consists of a lysis buffer which allow release of cellular caspases and a profluorescent caspase-3/7 consensus substrate, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide) rhodamine 110 (Z-DEVD-R110). Upon cleavage on the C-terminal side of the aspartate residue in the DEVD peptide substrate sequence by caspase-3/7 enzymes, the rhodamine moiety is released and able to fluoresce at 521nm when exited at 498nm. Since caspases exhibit substrate specificity for a 4 residue aspartic acid containing sequence in their substrate proteins the reagent is selectively activated by caspases 3 and 7 (Smolewski et al., 2002).

Before carrying out the assay, microplates were set up containing 100μM etoposide and TFA lunacridine (the highest concentration used in the assay) to check that neither drug fluoresced or absorbed at the specified wavelengths. The reagent was made up according to manufacturer's instructions by diluting the rhodamine substrate 1:100 with the lysis buffer and warmed to 37C° in a water bath. After addition of the Apo-1 reagent the plates were shaken using the Wallac Victor 1420 multi label counter and then incubated for 7 hours on a bench top with the lid on before reading fluorimetrically at 498nm excitation and 521nm. Graphs were then plotted as % increase in fluorescence over no

drug controls. Because of the expense of the reagent the experiment was first carried out at a fixed concentration (100μM) to find out which cell lines/drugs increased caspase activity and at what time interval. As with previous experiments each data point (well) was replicated eight fold.

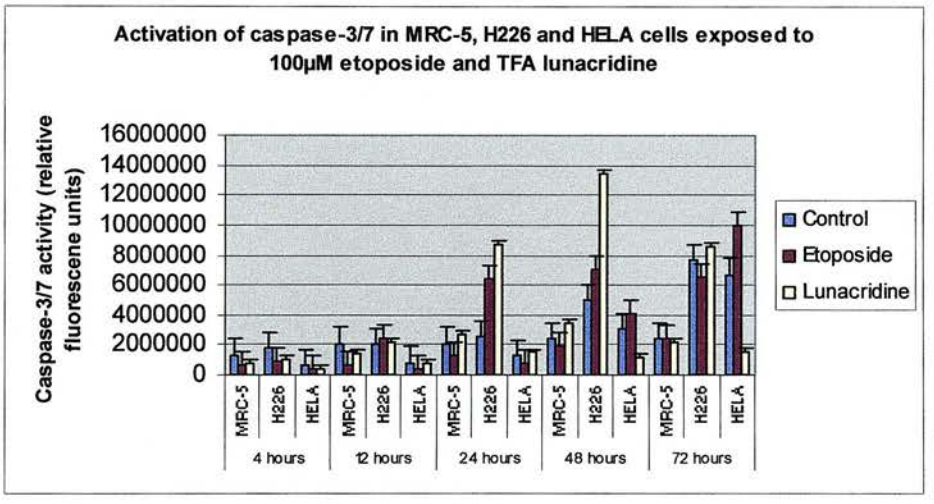


Figure 5.16 Activation of caspase-3/7 activity in H226, MRC-5 and HELA cells with increasing exposure to TFA Lunacridine and Etoposide. Cells were trypsinised and plated into opaque white 384 well plates and allowed to attach overnight before adding drugs. The plate was then incubated for the prescribed time intervals before addition of Apo-1 reagent and shaking for 1minute. The plate was then incubated at room temperature for a further 7 hours before reading fluorimetrically in a plate reader Ex 498 Em 521.

The results show a marked increase in caspase 3/7 activity with the H226 cells for both lunacridine and etoposide between 24 hours and 48 hours. There is also a gradual increase in caspase 3/7 activity with time suggesting that as the cells become serum starved and/or confluent they naturally undergo apoptosis. To further investigate the effects of the two drugs on H226 cells the experiment was repeated at increasing drugs concentrations from 12.5 to 100μM (Figure 5.17).

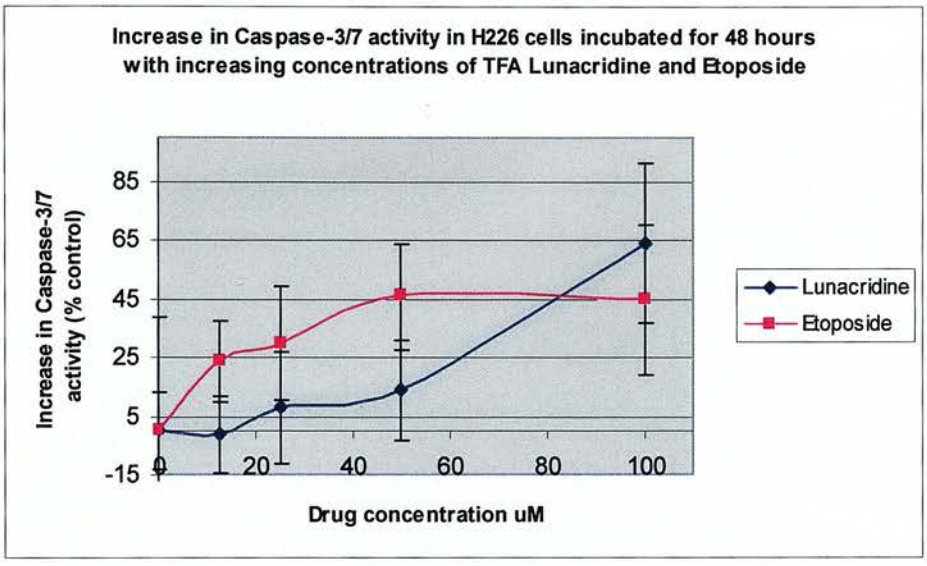


Figure 5.17 Activation of caspase-3/7 activity in H226 cells with increasing concentrations of TFA Lunacridine and Etoposide after 48 hours exposure. Activity is expressed as % increase over no drug controls. Cells were trypsinised and plated into opaque white 384 well plates and allowed to attach overnight before adding drugs. The plate was then incubated for 48 hours before addition of Apo-1 reagent and shaking for 1minute. The plate was incubated at room temperature for a further 7 hours before reading fluorimetrically in a plate reader Ex 498 Em 521. Cell viability is expressed as a percentage of a no drug control and each drug concentration was repeated 8 times.

Chapter 6

DISCUSSION

6.1 Ethnobotanical fieldwork

The ethnobotanical survey provided a list of over 200 plant species many of which were used in an antiseptic type-role but despite this only three species exhibited clear antibacterial activity against *E. coli* and *S. aureus* in the field screening process. Several different factors are likely to have contributed to this, ranging from selective antibacterial activity, non-antibacterial modes of actions (such as wound healing activity/adhesion inhibition mechanisms) and variations in the levels of active plant constituents.

An inherent limitation of the field screening approach was that only non-fastidious, non-pathogenic organisms would suit the rapid testing requirements of the assay kit. Thus plants that exhibited a broad spectrum of antibacterial activity stood the best chance of detection. This is unfortunate since a narrow spectrum of activity may be indicative of a highly specific mode of action, something often lacking with plant-derived compounds. High molecular weight molecules such as polyphenolics and tannins that lack drug-like properties are notorious for giving positive results in assays by virtue of their ability to bind covalently to proteins via their numerous hydroxyl groups (Cowan, 1999). The assay could therefore be improved by including a wider range of strains representing several bacterial genera to increase the likelihood of detecting the more selective antibacterial plants.

A second factor that was likely to have reduced the number of hits from the screening process is the presence of plants with non-antibacterial mode of actions. Inhibition of bacterial cell adhesion has been shown to be an effective means of suppressing infection (Sharon and Ofek, 2002) since adherence of gram-positive species such as *S. aureus* to endothelial cells is a prerequisite for the tissue-invasive stage of bacterial infection (Fischetti, 2000; Kuusela, 1978) whilst agents that inactivate bacterial toxins and promote the host immune response have also shown promise (Cazzola et al., 2004). Plants whose active constituents worked through either of these mechanisms would of

course not have been detected with the field assay. The role of insect vectors in infections such as the tropical ulcer must be considered too. The tropical ulcers described by informants in the field were observed to be attractive to flies which would attempt to feed on the ulcerative tissue. Spirochaete bacteria are known to be transmitted by the fly *Hippelates pallipes* and remain motile in its pharynx and oesophageal diverticula for up to several hours whilst *Hippelates* flies have been observed feeding on purulent skin lesions in Panama (Allen and Taplin, 1974). The use of insect repellent plants is well documented in the ethnobotanical literature (Seyoum et al., 2002; Waka et al., 2004) so it is plausible that their application to partially healed tropical ulcers, would reduce the chances of re-infection with Spirochaete bacteria.

Finally, variations in the levels of antibacterial plant metabolites present in individual plants, is likely to have had some impact on the efficacy of the screening process. Phytoalexins are defence compounds produced by the host plant in response to attack from plant pathogens (Baker et al., 1997) and many phytoalexins play a role in defending plants from pathogenic bacteria (Lugtenberg et al., 2002). Upon infection with plant pathogens, the plant detects the presence of the bacteria and activates specific host elicitor pathways, triggering their synthesis and greatly increasing their concentration in the affected plant tissue (Montesano, 2003). Another strategy employed by plants is to vary the concentration of defence compounds from leaf to leaf or between plants to make themselves an unpredictable food source. Studies with the neotropical tree *Cecropia peltata* reveal tannin levels may vary between trees by as much as 13mg to 58mg per gram of dry weight matter; it is thought that the presence of high tannin trees benefits neighbouring low tannin trees by making the population an unpredictable food source (Coley, 1986). Thus plants that displayed no antibacterial activity might have produced clear zones of inhibition had they been tested after undergoing some form of stress, or had been sampled from more than one plant.

6.2 Lunacridine's antibacterial activity

The baseline resolution HPLC separation of the *Lunasia amara* crude extract using a 300mM KCl mobile phase showed lunacridine to be almost entirely responsible for the anti-*S. aureus* activity of the bark extract with a very minimal contribution from peak 3 which was not identified (Figure 4.43). Since the bark extract was not subjected to any preliminary cleanup step, the HPLC running conditions were mild and the extract prepared from a specially non-dried sample it is unlikely that active principles were lost or chemically inactivated. Lunacridine is the major bark alkaloid comprising some 10% of the extractable aqueous bark solids or approximately 0.014% of the overall bark in disagreement with Johnstone et al 1958 who reported lunacrine as the major component (0.5-1%) (Johnstone, 1958). Johnstone et al were working with samples of the Australian variety however, (referred to as *L. quercifolia*) and assuming the alkaloidal composition of the species were to reflect the extreme variation in vegetative features reported by Hartley (1967) such a difference should be expected (Hartley, 1967).

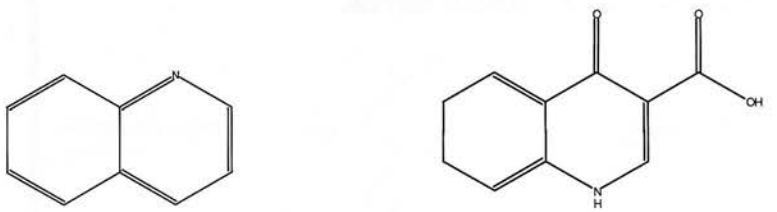
Overall, the antibacterial activity of lunacridine was disappointing; with an MIC for *Staphylococcus aureus* NCTC 6571 of 64μg/ml it is considerably less active than clinically used antibiotics most of which exhibit MICs for their target organism of less than 1μg/ml. Furthermore, considering its activity against human cells it is unlikely the compound could be considered a potential lead for antibiotic development. Based on lunacridine's topoisomerase II inhibition activity prokaryotic homologues of the enzyme such as DNA Gyrase or topoisomerase IV seemed potential targets of the drug in bacteria. To test this theory the compound was assayed on disks against several strains of *Streptococcus pneumoniae* (Table 5.2). The resulting zones of inhibition showed a clear pattern of activity which correlated with resistance to the quinolone antibiotic ciprofloxacin. Thus there was a decrease in the lunacridine zone of inhibition with strain

3018 which displays a raised MIC value against the quinolone antibiotic ciprofloxacin. The zones size did not alter accordingly for the other two strains (25306, SZ 27033) even though their MICs against erythromycin and penicillin were considerably greater than 3018 for ciprofloxacin. This demonstrates that both ciprofloxacin and lunacridine anti- *S. pneumoniae* activity are affected by the same drug resistance mechanisms thus raising the possibility of a shared mode of action between the two drugs.

Other mammalian topoisomerase II poisons and intercalators were tested for antibacterial activity to see if there is a general pattern of cross over between the eukaryotic and prokaryotic topoisomerase targeting drugs (Table 5.1). The clinically used anti-tumour compound etoposide showed a similar pattern of activity to lunacridine, with weak activity for *S. aureus* (32μg/ml) but no activity for *E. coli*. This is in line with reports of a related topoisomerase poison mode of action between the two drugs in bacteria (Sioud and Forterre, 1989). Chloroquine however which is a highly efficient DNA intercalator did not show any antibacterial activity. It is unclear why lunacridine and etoposide inhibit *S. aureus* but not *E. coli* whilst nalidixic acid, which shares some characteristics with lunacridine is far more active against *E. coli* than *S. aureus*. A possible clue however can be gained from the mode of action of the fluoroquinolones which exhibit a 1000 fold increase in potency towards certain gram positive bacteria over non-fluoronated quinolones such as nalidixic acid (Anderson et al., 1999). In gram positive bacteria topoisomerase IV is the primary target of fluoroquinolones as opposed to Gram negative species where Gyrase is more important (Ferrero et al., 1994; Maxwell, 1997). Whilst both enzymes are type II topoisomerases, bacterial topoisomerase IV is more analogous to its mammalian counterpart in that it is able to deconcatermerise chromosomes and resolve knots in DNA as opposed to DNA gyrase whose role is more centred on controlling superhelical density (Anderson et al., 1999). Thus it is possible that lunacridine and etoposide selectively target topoisomerase IV of gram positive bacteria. The lack of activity of chloroquine is less surprising since DNA intercalation is not by itself sufficient to produce cytotoxicity.

6.3 Lunacridine and its DNA intercalation activity

Upon identifying lunacridine as the active ingredient in the bark extract it was clear that it shared some structural similarities to certain quinolone and quinoline drugs. The quinolines and quinolones are both bicyclic nitrogen containing compounds but in the quinolones a carbonyl group is directly opposite the ring nitrogen at position 4 and a carboxylic acid side chain directly adjacent to the carbonyl group at position 3 (Figure 6.1) (Park et al., 2002). Both are extremely important pharmacaphores which form the basis of numerous anti-infective agents such as antibiotics (Domagala, 1994), antimalarials (Chong and Sullivan, 2003), anti-virals (Richter et al., 2004) as well as cytotoxic tumour drugs (Chu et al., 1994). What they share in common is an ability to interact with DNA and so inhibit the activity of certain DNA associated enzymes. In the case of the quinolones the DNA interaction occurs through non-specific groove binding (Park et al., 2002) whereas the quinolines tend to intercalate between base pairs by virtue of their planar structure (Yin et al., 2003).



Quinoline Quinolone

Figure 6.1. Quinoline and quinolone pharmacaphores.

As a planar quinoline is seemed likely lunacridine would act as a DNA intercalator and the results of the ethidium bromide DNA fluorescence assay confirm this. TFA lunacridine intercalates with an IC_{50} of approximately 0.2mM; somewhat less active than chloroquine and amsacrine but more so than nalidixic acid which did not show any intercalation activity.

DNA intercalators are molecules that insert themselves between base pairs in the DNA double helix (Brana et al., 2001). The phenomenon was first investigated with the acridine dyes by L.S Lerman (Lerman, 1961) who postulated a mechanism in which untwisting of the double helix would provide sufficient space between the layers of bases to provide access to small planar heterocycles (Figure 6.2). The presence of a positively charged ring nitrogen increases the affinity for DNA (Snyder and Arnone, 2002) . In the common B-form of DNA the bases are in close Van der Waals contact so intercalation is not possible but by unwinding the double helix slightly, spaces of suitable depth are opened up between the layers of bases whilst leaving hydrogen bonding between the bases undisturbed (Lerman, 1961). In this way DNA intercalators are able to slot themselves between base pairs and by virtue of their aromatic structure participate in the base stacking π-π interactions thereby adding stability to the structure (Haq, 2002).

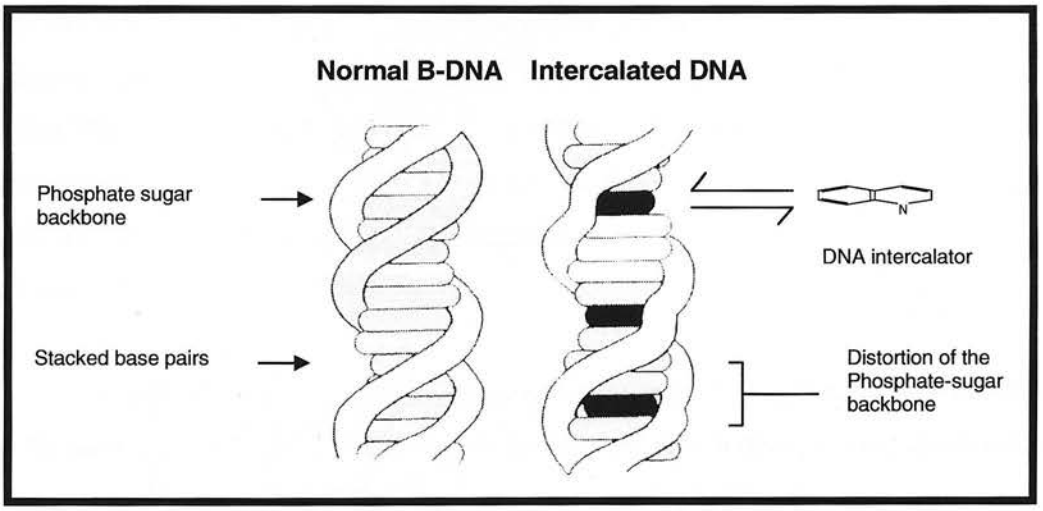


Figure 6.2. The Lerman model of DNA intercalation; changes in B-DNA structure upon intercalation of a planar cation between base pairs. To accommodate the intruding molecule the helix must unwind slightly causing a localised distortion of the phosphate-sugar back bone. (Adapted from Lerman et al (Lerman, 1961)).

Lunacridine exhibited mild intercalation activity compared with known intercalators such as the anti-neoplastic drug amsacrine and the antimalarial chloroquine which were approximately 20 to 40 times more active respectively (Figure 5.2). The prototype quinolone antibiotic nalidixic acid, did not exhibit any intercalating activity at all however, despite its apparent similarity in structure to lunacridine. It has been shown that DNA is a target of quinolone antibiotics such as nalidixic acid but the mode of interaction is not intercalative; the interaction being greater with single stranded than duplex DNA (Shen and Pernet, 1985). Studies with other quinolones such as norfloxacin suggest the drugs may partially intercalate into CpG steps as well as participating in non-specific groove binding, (Sandstrom et al., 2003) but this it seems is insufficient to displace intercalated ethidium Br.

Intercalation of both chloroquine and TFA lunacridine was considerably more efficient with calf thymus DNA than with the plasmid pBS. pBS like other plasmids is negatively supercoiled in its native state, and so intercalation of small molecules will result in a gradual relaxation of negative supercoiling with a concomitant reduction in superhelical writhe (Wang, 1974). Thus the lower IC_{50} for linear DNA than closed circular DNA could therefore be explained by differences in the forces that must be overcome to allow helix unwinding; plasmid unwinding being less energetically favourable due to the presence of higher torsional forces.

Intercalation of lunacrine was far less pronounced (Figure 5.2) and this can be attributed to the presence of the bulky furan ring on the alkaloid which disrupts the planar surface of the quinoline pharmacaphore. Samples prepared using KCl and mineral acids all displayed a lower efficiency of intercalation than those prepared with TFA and acetic acid; a phenomenon which can be explained by their propensity to cyclise whilst in storage. Reaction of the side chain hydroxyl to the ring carbonyl would produce a derivative identical to lunacrine except for a carbonyl group in the place of the methoxy group at position 4. This would create a furan ring and disrupt the planar structure of the alkaloid. Supporting this is the fact that proton NMR spectra recorded with underivitised

lunacridine samples showed an increase in impurities corresponding to lunacrine peaks after being stored at -20°C for several months (personal communication Dr I. Sadler).

6.4 Lunacridine and its topoisomerase II inhibition activity

It is well established that DNA intercalation, whilst not a prerequisite for eukaryotic topoisomerase II inhibition is nonetheless a related activity, since numerous DNA intercalators act as inhibitors of the enzyme (Brana et al., 2001). As suspected, TFA lunacridine proved to be an effective inhibitor with a mode of action that most likely falls within the topoisomerase poison category of inhibitors. Surprisingly however, complete inhibition of the enzyme occurred at 5μM (Table 6.1), well below that required for significant DNA intercalation, whereas by comparison the DNA tumour drug amsacrine gave complete inhibition at 1μM; a figure somewhat higher than its intercalative activity would have suggested.

Inhibitor	IC_{50} DNA intercalation (μM)	Complete inhibition of Topoisomerase II (μM)	Topo II inhibition/ intercalation
Amsacrine	4	1	0.250
TFA lunacridine	220	5	0.023

Table 6.1 IC_{50} concentrations for intercalation of amsacrine and TFA lunacridine with calf thymus DNA and concentrations at which topoisomerase II decatenation activity was inhibited.

6.4.1 Topoisomerase structure and function

Topoisomerase II is a highly conserved enzyme which is essential for normal cellular function (Roca, 1995). There are two mammalian isoforms of topoisomerase II, termed alpha and beta. The gene for topoisomerase II α is located on chromosome 17q21–22 and encodes a 170 kDa protein whereas the topoisomerase II ß gene encodes a 180 kDa

protein and is located on chromosome 3q24 (Kellner et al., 2002). They both have similar primary structures and exhibit virtually identical catalytic properties in vitro. Numerous lines of evidence suggest the alpha isoform to be the enzyme most involved with mitotic processes whereas the role of the beta isoform is more obscure (Sakaguchi and Kikuchi, 2004). In both isoforms, dimerisation of the gene product is required for topoisomerase activity.

The extreme length of genomic DNA poses several problems of access to nuclear proteins. As is the case with any string-like structures there is a natural tendency to form knots as well as to undergo torsional stress which if not overcome results in supercoiling, otherwise known as toroidal writhe (White and Bauer, 1986). This phenomenon is exploited by both prokaryotic and eukaryotic cells as a means of packaging genomic DNA but needs to be overcome to allow the myriad of DNA associated proteins such as polymerases to traverse the double helix. This role is in part carried out by the two isoforms of topoisomerase II which are able to untangle knotted DNA and relieve toroidal writhe. They do this by making double strand breaks in DNA and passing a segment of an adjacent DNA helix through the resulting gate before resealing and releasing the two segments (Kellner et al., 2002).

The precise mechanism by which topoisomerase II does this has been extensively studied and Topoisomerase II is the only enzyme capable of both cleaving and ligating double stranded DNA (Kellner et al., 2002). The enzyme is composed of two homodimers which when dimerised together form a DNA binding/ligation site along with gates to mediate the translocation of individual strands of DNA (Figure 6.3). The middle section is where double strand break formation and ligation occur while either side of this are the two gates formed from paring of the N and C-terminal portions of each dimer. A length of DNA termed the G-segment is cut in the middle section of the enzyme through interaction with two tyrosine residues on each monomer (Tyr-805 in human topoisomerase II alpha) (Jensen et al., 1996). A second piece of DNA termed the T-segment is then passed through the "gate" formed from the gap in the G-segment via opening and closing of the two N and C-terminal-formed gates. Where the G and T

segments are from different "links" in a concatemer, the enzyme cycle will mediate de-concatemerisation (Roca, 1995). The process can be summarised as follows:

1. The helix to be cleaved termed the G-segment is bound to the enzyme at a species dependent consensus sequence (Spitzner and Muller, 1988) where it undergoes nucleophilic attack at two different locations 4 base pairs apart producing a double strand break. The resulting 5´-phosphoryl groups at each side of the cut are held in place by covalent linking to two tyrosine residues on the enzyme. At this point a second DNA duplex termed the T-segment is free to pass in and out of the gate formed by the break in the G-segment.

2. Upon binding of ATP to the outermost section formed from the two N-terminal domains, the gate closes locking translocated T-segment on the opposite side of the enzyme.

3. The T-segment then exits the enzyme via a gate formed from the two C-terminal domains which then close behind it. The G-segment is then re-ligated, and upon hydrolysis of the bound ATP (which reopens the N-terminal gate) is released from the enzyme.

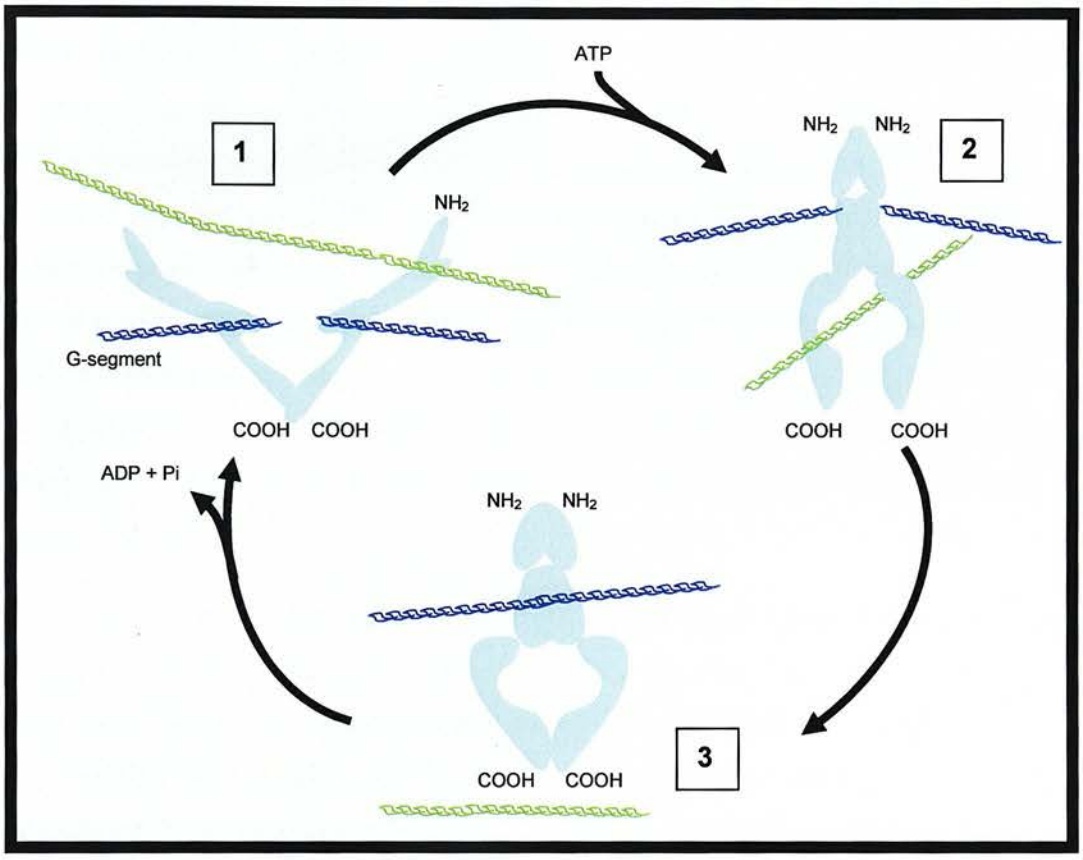


Figure 6.3. Mode of action of mammalian topoisomerase II. Adapted from (Roca, 1995).

6.4.2 Mode of action of topoisomerase II inhibitors

Inhibitors of type II topoisomerases may be divided into two classes namely catalytic topoisomerase inhibitors and topoisomerase poisons (Jensen and Sehested, 1997). The topoisomerase poisons act to stabilise the so called cleavable complex formed between the enzyme, drug and the transiently bound G-segment DNA (Larsen et al., 2003); drugs such as the epodophylotoxins (etoposide), the DNA intercalators such as amsacrine and the antibacterial quinolones all inhibit the enzyme via this mechanism. The catalytic inhibitors are typified by agents such as novobiocin and the coumermycins and act on

other steps in the catalytic cycle such as ATP binding (novobiocin), stabilise non-covalent DNA enzyme complexes (merbarone) or intercalate with DNA to prevent DNA enzyme interaction (aclarubicin) (Larsen et al., 2003). Despite the extreme variation in structure between antibacterial quinolones and eukaryotic topoisomerase II poisons (Huff and Kreuzer, 1990) and the limited sequence homology of their respective enzyme targets, (Wyckoff et al., 1989) there is still a degree of overlap in activities (Huff and Kreuzer, 1990; Yamashita et al., 1992). Indeed mapping of the drug interaction domain of *Drosophila* topoisomerase II demonstrates overlap in the enzyme drug binding site for structurally disparate inhibitors (Elsea et al., 1997). This is further exemplified by the antibacterial assays with etoposide and TFA lunacridine where both displayed anti-*S. aureus* activity.

TFA lunacridine and the clinically used topoisomerase poison amsacrine were studied using a topoisomerase II kDNA decatenation assay. The assay showed clear inhibition between 4μM-5μM TFA lunacridine and for amsacrine the effect occurred between 0.5 and 1μM in agreement with published figures. Thus despite amsacrine being approximately 100 fold more efficient a DNA intercalator the drug was merely 5-10 fold more potent an inhibitor of topoisomerase II. This phenomenon is mirrored by other topoisomerase poisons. Whilst some intercalators such as the acridine carboxamides (Figure 6.4) show a relationship between increased DNA affinity and topoisomerase II inhibition/cytotoxicity (Adams et al., 2000), the general consensus is that intercalation does not guarantee enzyme inhibition by itself.

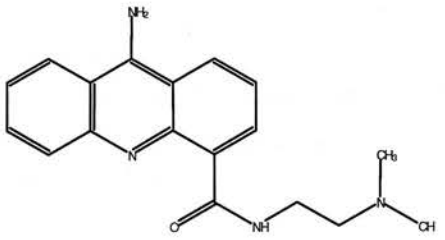


Figure 6.4. Acridine carboxamide compound 9-amino-DACA. The side chain is critical to its topoisomerase II inhibition activity.

Moreover, a side chain, capable of making contacts with the enzyme is required for activity. This is reflected in the acridine carboxamides structure activity relationship in which the side chain, which must be in the acridine 4 position and posses an unsubstituted NH group, two methylene groups and a terminal protonated *N,N*-dimethylamino group is a crucial feature (Adams et al., 2000). Thus it is the side chain which modifies the activity of the drug either by contacting the enzyme itself or making contacts with DNA that stabilise the cleavable complex (Capranico and Binaschi, 1998). A more dramatic example is provided by the two acridine based drugs *m*-AMSA (amsacrine) and *o*-AMSA which differ only in the position of a methoxy group (Figure 6.5). Whilst *m*-AMSA is a potent intercalator and topoisomerase II inhibitor *o*-AMSA displays only weak topoisomerase II inhibition activity and thus no cytotoxic activity despite a DNA binding affinity 4 times higher than M-AMSA (Pommier et al., 1987).

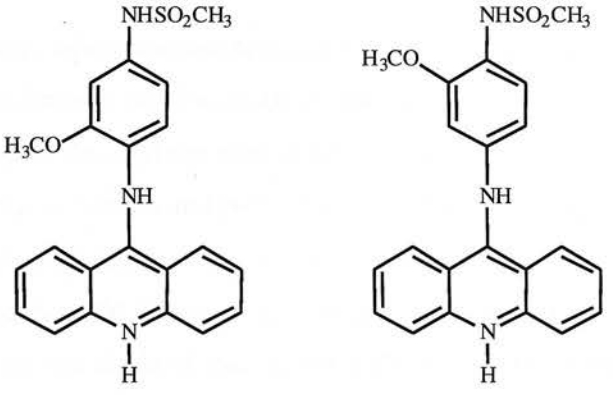


Figure 6.5. Structures of *o*-AMSA and *m*-AMSA which differ only in the position of a methoxy group. Unlike *m*-AMSA, *o*-AMSA does not posses topoisomerase II inhibition activity despite being the more efficient DNA intercalator of the two compounds.

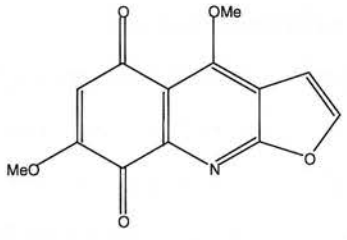
Various models have been put forward to describe the formation of the cleavable complex and these differ between the eukaryotic and prokaryotic forms of the enzyme.

Since crystal structures are not available for trapped topoisomerase II or gyrase, inferences on the position of DNA, drug and enzyme in the complex are predominantly based on mutation mapping studies carried out in bacteria, yeast and mammalian cell lines (Macinga et al., 2003). For the antibacterial quinolones the interaction with DNA is thought to be afforded either by a by a Mg^{2+} bridge which mediates binding of the drugs to DNA phosphate (Palumbo et al., 1993) or intercalation at the site of the cleaved phosphodiester bond (Freudenreich and Kreuzer, 1993). Structure-activity relationships suggest that position 6 of the quinoline pharmacaphore is involved with recognition of the gyrase enzyme pocket (Sissi et al., 1998) whilst numerous quinolone-resistance mutation mapping studies reveal locations near the N-terminal DNA binding domain such as Ser 83 of GyrA (gyrase) and corresponding residues of ParC (topoisomerase IV) as the likely location for contact between enzyme and drug (Ferrero et al., 1994; Yoshida et al., 1988).

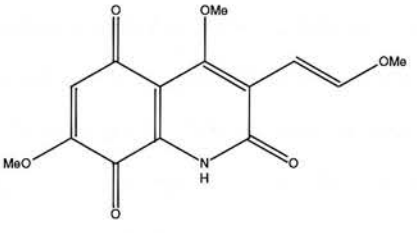
With eukaryotic topoisomerase II poisons such as amsacrine, intercalation with DNA occurs via the acridine portion of the molecule (Rene et al., 1996) whilst contacts with the enzyme occur through the substituent at position 1' on the anilino ring (Rene et al., 1997). Analysis of human and yeast topoisomerase II mutants reveal residues in the α4 helix of the enzyme as possible sites for drug interaction; intriguingly, one common yeast mutation Ser-740 is homologous with Ser 83 of prokaryotic DNA gyrase suggesting a similar mode of interaction to the quinolones with gyrase. (Strumberg et al., 1999). Molecular modelling of the amsacrine cleavable complex carried out using crystal co-ordinates from a large fragment of yeast topoisomerase II (Berger et al., 1996) suggests a model in which the acridine chromophore is intercalated at the site of the cut G-segment with two of the methyl hydrogens from the $NHSO_2CH_3$ group of the anilino ring forming hydrogen bonds with Thr 744 and Gly 747, both of which are adjacent to Ser 740 in the DNA binding domain of the enzyme (Moro et al., 2004). Thus for *o*-AMSA the configuration of the side chain does not support contacts with these residues, so explaining its inability to stabilise the cleavable complex.

It is therefore possible that the lunacridine side chain performs a similar role and accounts for the unexpectedly potent topoisomerase inhibitory activity of the drug relative to its intercalative ability. Assuming the molecule were to form a cleavable complex analogous to that described for amsacrine (Moro et al., 2004) a perplexing result is that trifluoroacetylation of the side chain does not eliminate topoisomerase II inhibition activity suggesting the side chain hydroxyl is not involved with enzyme interaction. It is tempting to postulate a model in which lunacridine intercalates at the site of strand cleavage and contacts residues in the vicinity of those involved with amsacrine binding via its T-butyl methyl hydrogens or those of the methoxy groups.

Several quinolones bearing structural similarities to those of *Lunasia amara* have been purified from the bark of a related species *Sarcomelicope megistophylla* Hartley (Rutaceae). Megistoquinone II (Figure 6.6) has a similar bicyclic structure to lunacridine with a side chain at position 3 whilst megistoquinone II is analogous to lunacrine in that the side chain is used to close a furan ring with the carbonyl at position 2. Megistoquinone II has an MIC of 0.75mg/ml for *S. aureus* ATCC 25923 compared with 2.35mg/ml for megistoquinone I. Thus whilst both compounds are only weakly active, megistoquinone II with its planar structure and side chain is the more active compound further exemplifying the importance of such features (Fokialakis et al., 2001).



Megistoquinone I



Megistoquinone II

Figure 6.6. Structures of megistoquinones I and II from *Sarcomelicope megistophylla* (Rutaceae)

Once topoisomerase II had been established as the target of lunacridine, a further experiment was carried out to probe the mode of enzyme inhibition. Amsacrine and lunacridine were pre-incubated with the enzyme at twice the concentration required for total inhibition of decatenation (2μM and 10μM respectively) (Figure 5.7). kDNA and reaction buffer were not included so DNA intercalation and ATP binding could not occur. After incubation at 37°C for 10 minutes, water, reaction buffer and kDNA substrate were added diluting the drug 10 fold to a concentration below that required for inhibition to occur. After further incubation for 1 hour at 37°C the reaction products were analysed revealing no inhibition to have taken place. The result suggests DNA must be present for enzyme inhibition to occur and precludes a covalent enzyme binding mode of action for both drugs. This is consistent with amsacrine's established mode of action as a topoisomerase poison. A catalytic inhibitor mode of action for lunacridine cannot be ruled out however since drugs such as the anthracycline aclarubicin act as catalytic inhibitors by intercalation alone and without stabilising a cleavable complex (Larsen et al., 2003). This would appear unlikely for lunacridine however, since topoisomerase II inhibition occurred at concentrations below those required for significant DNA intercalation.

6.5 Tumour cell inhibitory activity and Caspase 3/7 activation

The interaction of lunacridine with DNA and its low micromolar inhibition of topoisomerase II made it likely it would show some degree of cytotoxicity towards human cells. Although this did not relate directly to the ethnobotanical use of *Lunasia amara*, such a pattern of activity is in keeping with drugs like novobiocin and etoposide which inhibit both bacteria and mammalian cells due to concomitant prokarytotic/eukayotic type II enzyme inhibition activity (Larsen et al., 2003; Sioud and Forterre, 1989).

Topoisomerase II poisons are known to cause double strand breaks in cellular DNA (Jensen and Sehested, 1997) and if such breaks are not repaired they may trigger apoptosis (Kaina, 2003; Sordet et al., 2003). This activity is exploited by cytotoxic cancer drugs such as amsacrine and etoposide which are able to produce selective killing of tumour cells (Dolega, 1998). It is thought that such selective cytotoxicity is possible because cancer cells divide more rapidly than healthy cells and so undergo replication more frequently (Marchini et al., 2004); the high topoisomerase II activity of tumour cells resulting in increased DNA damage (De Beer et al., 2001; Holden, 2001; Lock and Ross, 1987)

Apoptosis is distinct from cellular necrosis in that it represents a programmed biochemical process which takes time to complete whereas necrosis on the other hand occurs rapidly after the initial insult and is not a programmed event (Philchenkov, 2004). Caspases (cysteinyl aspartate-specific proteases) are key effector molecules in the apoptotic pathway and are divided into two groups namely effector and initiator caspases, 12 of which have been identified so far (Philchenkov, 2004). Initiator caspases which include caspases 2, 8, 9, 10 and probably 11 activate the effector caspases 3, 6 and 7 (Wolf and Green, 1999). Initiator step pathways are grouped around key organelles such as mitochondria, golgi apparatus, the nucleus as well as cell membrane receptor activated pathways (Ferri and Kroemer, 2001). Once activated the effector caspases are able to cleave a wide range of substrates which include crucial structural and regulatory proteins thereby destroying the cell (Liu et al., 2005). DNA damaging agents may activate both the membrane death receptor pathways (Beck et al., 2001) and the endogenous mitochondrial damage pathway through upstream events that occur in the nucleus as a result of DNA lesions (Kaina, 2003).

The cascade of events that lead to topoisomerase II poison induced apoptosis begins with the formation of the cleavable complex which consists of drug, DNA and topoisomerase enzyme trapped mid way through its catalytic cycle (Larsen et al., 2003). Topoisomerase II is covalently attached to cleaved DNA and unable to complete its

catalytic cycle due to the presence of the topoisomerase poison which contacts both the DNA and enzyme (Binaschi et al., 1995). The cleavable complex is not lethal in itself but is converted into DNA double strand breaks during replication and transcription when enzymes such as helicase collide with it, dissociating topoisomerase II to reveal a double strand break (D'Arpa et al., 1990; Howard et al., 1994).

Detector protein kinases DNA-PK, ATM and ATR bind preferentially to DNA breaks and activate downstream kinases such as c-Abl and the checkpoint protein Chk2 which induce apoptosis by activating cell death pathways such as SAPK, p53 and p73 as well as inhibiting certain cell survival pathways (Kaufmann, 1998; Sordet et al., 2003). P53 is a key agent in the activation of apoptosis in that it may send a cell into cell cycle arrest to allow repair of genetic lesions since it possesses 3´-5´ exonuclease function as well as an ability to detect and prevent erroneous homologous recombination (Clifford et al., 2003; Valkov and Sullivan, 2003). If repair of the lesion is not carried out correctly it activates a wide range of pro-apoptotic signalling molecules (Valkov and Sullivan, 2003). Further evidence for p53's role in DNA damage induced apopotosis comes from tumour cells expressing defective p53 which are resistant to topoisomerase II inhibitors such as etoposide (Valkov and Sullivan, 2003).

There is a high degree of cross-talk between topoisomerase II and p53 which both binds and stimulates the catalytic activity of the enzyme as well as controlling its expression (topoisomerase II α only) by binding to a promoter upstream on its gene (Valkov and Sullivan, 2003). Recent evidence suggests DNA damage may also result in caspase-2 being recruited to a complex termed the PIDDosome which contains the death domain protein PIDD and the adaptor protein RAIDD (Tinel and Tschopp, 2004). Caspase-2 is a key initiator of the mitochondrial death pathway which it activates by stimulating the release of cytochrome C (Philchenkov, 2004). Thus once DNA double strand breaks reach a critical level, and are beyond repair, a set of events is set into motion which lead to the induction of apoptosis and eventual cell death.

The gradual decline in cell viability seen with the Cell Titer reagent was both time and concentration dependent in line with what would be expected from topoisomerase II induced apoptosis. At 4 and 12 hours there is very little difference between the cell types but at 24 hours (Figure 5.12)TFA Lunacridine starts to show marked inhibition of both H226 and MRC-5 fibroblasts. H226 is the more inhibited of the two between 25 and 50 μM but MRC-5 more so at concentrations over 100μM. This is in contrast to etoposide which shows little inhibition of any cell line at 24 hours. At 48 hours (Figure 5.13) MRC-5 is inhibited more than other cell lines by lunacridine whereas it is unaffected by etoposide which starts to show selectivity for H226. At 72 hours (Figure 5.14) etoposide shows selective inhibition for H226 and HELA inhibiting the faster growing HELA cells slightly more than H226 whilst the less proliferative MRC-5 is completely uninhibited and indeed shows a marked increase in cell viability in response to increasing etoposide concentrations. This selectivity is not matched by lunacridine which shows only slightly more inhibition of H226 and HELA than MRC-5 especially at the lower concentration ranges. Of the two compounds TFA lunacridine was the more active with 50% inhibition at 72 hours occurring at less than 12.5μM as opposed to etoposide which failed to reach 50% inhibition. The overall lack of selectivity of lunacridine between the tumour cell lines (H226 and HELA) and the non-carcinoma MRC-5 fibroblasts is shown by the plot of cell viability at 12.5μM over time (Figure 5.15) in which there is little difference in cell viability between the three cell types over the full 72 hour time course. This compares unfavourably with etoposide which shows an approximate 40% to 60% difference in cell viability between H226/MRC-5 and HELA/MRC-5 respectively. Thus on the basis of the cell viability experiments alone, TFA lunacridine does not posses sufficient selectivity to merit its development as an antitumour compound. The structural basis for the selectivity of compounds such as etoposide remains unclear but it is thought that sequence specific binding may play a role (Palumbo et al., 2002). Thus it is possible that the right structural modifications to the lunacridine pharmacaphore might impart selectivity for carcinoma cell lines.

At both 48 and 72 hours there is a surprisingly non-linear relationship between drug concentration and cell viability which is exemplified by lunacridine with HELA cells but is also seen with etoposide. The cell viability curve shows an initial sharp decrease between 0 and 12.5μM after which point cell viability increases with lunacridine concentration so that at 100μM cell viability is 60% despite being nearly 20% at 12.5μM. Such a non-linear dose response curve is characteristic of topoisomerase poisons such as the antrhacyclines and ellipticines whose ability to promote DNA strand breakage follows a bell shape curve starting with increasing activity and then declining with increasing drug concentration (Koo et al., 1999; Pommier Y, 1996). It is thought that at high drug concentrations, intercalated drug occupies so many DNA binding sites that it obscures the topoisomerase binding site (Liu, 1989). Such "template blockage" prevents the stabilisation of the cleavable complex and therefore the formation of DNA strand breaks and the induction of apoptosis. Thus lunacridine acts as an inhibitor of its own cytotoxic activity at high concentrations. This distortion of the expected monotonic curve further supports a topoisomerase poison mode of action for lunacridine.

The caspase 3/7 experiments indicated caspase activation in H226 cells with both of the drugs between 24 and 48 hours exposure (Figure 5.16). Lunacridine is the more effective of the two with more than four fold increase in caspase activity at 24 hours. This coincides with the decrease in H226 viability seen at 24 and 48 hours. Thus on the basis of caspase 3/7 activity alone, TFA lunacridine is a highly selective inducer, more so even than etoposide showing almost no induction of caspase 3/7 with MRC-5 at any of the time intervals. Perplexingly, neither agent displayed any such activity towards HELA cells but this may be due to the narrow window of activation of caspases which may have been missed by the choice of time intervals. Activation of caspase 3 and 7 in H226 cells was repeated with varying drug concentrations (Figure 5.17) achieving a similar result but with etoposide the more effective inducer at lower concentrations. At 48 and 72 hours there is marked increase in caspase 3/7 activity of all the cell lines including the controls suggesting that serum depletion of the cells had induced apoptosis.

6.6 Anti HSV-1 and reverse transcriptase inhibitory activity

6.6.1 Anti HSV-1 activity

Various lines of evidence pointed towards a potential anti herpes simplex virus (HSV) activity for lunacridine. Firstly, ethnobotanical data on the use of *L. amara* describe its use in the Philippines to treat inflamed eyes (Hartley, 1967), a description that closely resembles conjunctivitis one of the causative agents of which being HSV-1 (Stumpf et al., 2002). Secondly, HSV-1 replication has been shown to be dependent on cellular topoisomerase II (Bailly, 2000) (HSV-1 does not encode its own copy of the enzyme) and inhibition of topoisomerase II using classical anti-tumour compounds such as amsacrine and etoposide has been shown to inhibit the replication of cytomegalovirus (another member of the Herpesviridae)(Benson and Huang, 1988).

The TFA lunacridine sample was used in a virus yield reduction assay against a strain of HSV-1 (SC16) but failed to show any degree of inhibition. This was unexpected considering recent reports in the literature of the potential for topoisomerase II inhibitors as anti-HSV agents (Bailly, 2000). Crosslinking studies have shown that the alpha isoform (p170) associates with the viral DNA (Ebert et al., 1994) and since lunacridine was shown to inhibit this isoform it should be expected to also inhibit viral replication. It is not certain however that inhibition of topoisomerase II is responsible for the antiviral action of amsacrine and etoposide and the possibility remains that their antiviral action occurs through interaction with a second target that is distinct from cellular topoisomerase II. Evidence to support this idea comes from the 7-substitueted-1,3-dihydroxyacridones which were originally synthesised as DNA intercalating topoisomerase II inhibitors and subsequently screened for anti-HSV activity, revealing the 7-chloro derivative to be highly active. But more recent work on this class demonstrates that topoisomerase II is unlikely to be the target of the drugs (Akanitapichat et al., 2000) and that inhibition of HSV-1 DNA polymerase is more likely to be responsible for their antiviral activity (Bailly, 2000).

6.6.2 Reverse transcriptase inhibition activity

Various quinolines and quinolones have been shown to be active against retroviruses including chloroquine which has recently emerged as a potent HIV-1 inhibitor (Boelaert et al., 2001). Other intercalators such as doxorubicin are known to inhibit mouse leukaemia virus reverse-transcriptase (Tomita and Kuwata, 1976) whilst nalidixic acid is an inhibitor of avian myeloblastosis virus reverse-transcriptase, all be it at relatively high concentrations (Aoyama, 1991). Furthermore, studies with the DNA intercalating benzo[*c*]phenanthridine alkaloid fagaronine isolated from the roots of another species of Rutaceae has demonstrated its activity against a number of reverse transcriptases from different sources (Fleury et al., 2000).

An ELISA based assay was used to determine the reverse transcriptase inhibitory activity of TFA lunacridine along with nalidixic acid and chloroquine. The results place lunacridine as the most active of the three compounds with an IC_{50} of approximately 1.25mM (Table 6.2). Nalidixic acid was the least active with an IC_{50} of 17mM whilst chloroquine's IC_{50} was approximately 2mM. Thus as was the case for topoisomerase II inhibition the efficiency of DNA intercalation does not correlate with enzyme inhibition activity. This would seem to rule out a mechanism in which the compounds inhibit reverse transcriptase simply as a result of their intercalation with DNA since if this were the case chloroquine would be significantly more active than lunacridine. Unfortunately IC_{50}'s are far higher than the level at which cytotoxicity was detected in human cell lines so lunacridine could not be assayed against HIV-1 in vivo.

Inhibitor	IC_{50} DNA intercalation (μM)	IC_{50} RT inhibition mM	RT inhibition / Intercalation
Chloroquine	14	1.4	0.1
Nalidixic acid	> 660	17.2	< 0.03
TFA lunacridine	220	1.3	0.006

Table 6.2 Comparison of RT inhibition activity and calf thymus DNA intercalation activity between chloroquine, nalidixic acid and TFA lunacridine.

6.7 Conclusion, *Lunasia amara* as a plant medicine

The results of the various assays can be interpreted to determine the efficacy of the plant medicine in terms of its intended use i.e. the treatment of tropical ulcers. The plant was prepared by scrapping small amounts of material from the surface of the bark so as to produce fine shavings which were then applied directly to the tropical ulcer. The fresh bark was moist and this presumably aided the transfer of lunacridine to the site of infection. Since un-concentrated aqueous extracts of the bark give sizeable zones of inhibition with *S. aureus* (which is thought to be involved with the ulcerative process), (Braun-Falco, 1991) it would seem likely that the bark would have a therapeutic effect. This would occur despite the relatively high MIC of lunacridine for *S. aureus* (64 μg/ml) since the bark appears to be particularly rich in the alkaloid. The hydrophilic nature of lunacridine would also aid its transfer and solubility in the aqueous environment of the ulcer. Thus the efficacy of a plant derived ethnomedicine such as *Lunasia amara* bark cannot be determined solely by the activity of purified active constituents but would be better described in terms of the activity of the whole bark as it exists in the field. Furthermore, as has been seen through the numerous attempts at purification, the pure alkaloid is not stable in its pure form but when applied direct from the tree bark it would easily reach the site of infection intact.

A potential problem with using a DNA intercalating topoisomerase II inhibitor such as lunacridine as a topical antibacterial agent is that it provides little selectivity for the bacteria in the wound over the cells in the infected tissue. It is not possible to determine the concentration of lunacridine that would reach the site of infection since the alkaloidal content would vary from tree to tree but since the MIC for *S. aureus* is greater than the IC_{50} for MRC-5 human cell viability (< 5μg/ml after 72 hours incubation) the bark preparation would be expected to impart a certain degree of toxicity to the infected tissue.

DNA intercalators such as lunacridine are potentially mutagenic since by inserting between bases there is a tendency for polymerases to recognise them as a purine base and by inserting a corresponding pyrimidine give rise to a frame shift mutation (Hoffmann et al., 2003). Secondly, topoisomerase inhibition which results in double strand breaks i.e. topoisomerase poisoning is a potentially mutagenic event (Boos and Stopper, 2000) since repair of the double strand breaks or action of exonucleases on the exposed ends of DNA may result in deleterious repair and loss function mutations. There is even evidence to suggest that etoposide induces chromosomal recombination during meiosis since re-ligation of double strand breaks may result in rejoining of the original un-crossed strands (Russell et al., 2000). Mutagenic effects arising from the application of *L. amara* bark to tropical ulcers would most likely go unnoticed however since any carcinogenic effect would occur long after the event.

Despite this, in evaluating the use of the plant preparation it is necessary to way the cost of leaving an infected wound untreated against the likely impact of possible side effects. Since tropical ulcers have the potential to develop into chronic infections which may ultimately result in amputation or septicaemia (MacDonald, 2003), and antibiotics are not readily available to the inhabitants of the Whiteman Range in Papua New Guinea; the use of *L. amara* bark may well represent a worthwhile risk.

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